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Endogenous, Exogenous and Novel Allosteric Modulators of Ligand-Gated Ion Channels

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Endogenous, Exogenous and Novel Allosteric Modulators of Ligand-Gated Ion Channels

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Dedication

I dedicate this dissertation to my family, whose support has made this chapter in my life possible.

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Endogenous, Exogenous and Novel Allosteric Modulators of Ligand-Gated Ion Channels

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The central nervous system is a web of neuronal communication, mediated through excitatory and inhibitory signals. These signals are mediated in part by the amino acid neurotransmitters glutamate, GABA and glycine, which are ligands for specific ligand-gated ion channels. While direct activation or inactivation of ligand-gated ion channels can cause marked and sometimes detrimental changes in the nervous system, allosteric modulators of these channels can produce more subtle and specific changes. Despite the clinical potential of allosteric modulators, the molecular mechanisms of their action at ligand-gated ion channels are poorly understood. This dissertation covers several aspects of allosteric modulation: elucidating the molecular mechanism of known exogenous modulators, identifying endogenous compounds as modulators, and using a drug discovery technique to find novel modulators. The first chapter details how ketone bodies are endogenous allosteric modulators of NMDA, $GABA_A$ and glycine receptor function, and that this action of ketone bodies may underlie therapeutic benefits. Next, I characterize how benzodiazepines, acting as positive allosteric modulators of the $GABA_A$ receptor, produce a molecular rearrangement in the $GABA_A$ receptor resulting in an enhancement in receptor function. This involves an interaction between charged residues at the interface of adjacent subunits of the $GABA_A$ receptor. Finally, I utilize phage display technology to identify peptides capable of

allosteric modulation of the $GABA_A$ receptor, providing evidence that this technique can be used to identify new compounds for a large range of indications.

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CHAPTER 1. INTRODUCTION

1.1 Amino Acid Transmitters: Glutamate, GABA and Glycine

Amino acid transmitters are amino acids capable of participating in synaptic transmission, and can be divided based on their excitatory or inhibitory properties. Glutamate, gamma-aminobutyric acid (GABA) and glycine are the most prevalent amino acid transmitters, with glutamate acting in an excitatory manner and GABA and glycine acting in an inhibitory manner via their respective receptors. These amino acids are natural metabolites produced by a variety of pathways.

Glycine is produced from another amino acid, serine, by the enzyme serine hydroxymethyltransferase while glutamate and GABA come indirectly from the breakdown of glucose via the Krebs cycle. α-ketoglutarate, a Krebs cycle intermediate, is converted to glutamate via alanine transaminase. Glutamate can also be synthesized from another amino acid, glutamine, found in astrocytes at high concentrations. Astrocytes take up extracellular glutamate via excitatory amino acid transporters, and convert glutamate to glutamine using glutamate synthase. Glutamine can exit astrocytes and enter neurons (both via glutamine transporters), where glutaminase can re-convert glutamine to glutamate. Glutamic acid decarboxylase converts glutamate to GABA.

After synthesis, glutamate, GABA and glycine are packaged into distinct synaptic vesicles, sometimes within the same neuron (Todd et al., 1996; Somogyi and Llewellyn-Smith, 2001; Gundersen, 2008; Shabel et al., 2014). Depolarization of a presynaptic neuron triggers calcium ion influx through calcium channels which in turn results in fusion of the synaptic vesicle with the

presynaptic membrane, releasing the synaptic vesicle contents into the extracellular space where they can then act on their respective presynaptic and/or postsynaptic receptors.

1.2 Neurotransmitter Actions on a Receptor

The binding of a neurotransmitter (agonist) to its receptor triggers a conformational change in the receptor which results in a subsequent event, such as the opening of an ion-conducting pore or G-protein activation. The ability of an agonist to bind to its receptor is determined by the affinity of the agonist for that receptor. The ability of the agonist, after binding, to produce subsequent conformational changes leading to receptor activation, is determined by the agonist's efficacy for the receptor. These separate affinity and efficacy steps of drug action at a receptor were first described by del Castillo and Katz in 1957. (**Figure 1**). While the binding step can be maximized (as is seen in 100% binding site occupation in the case of irreversibly-binding ligands), efficacy has no upward limit since it is a ratio of two rate constants that describe the transitions between closed and open states of ion channels; the term full agonist is sometimes used to describe the most efficacious compound known at that time.

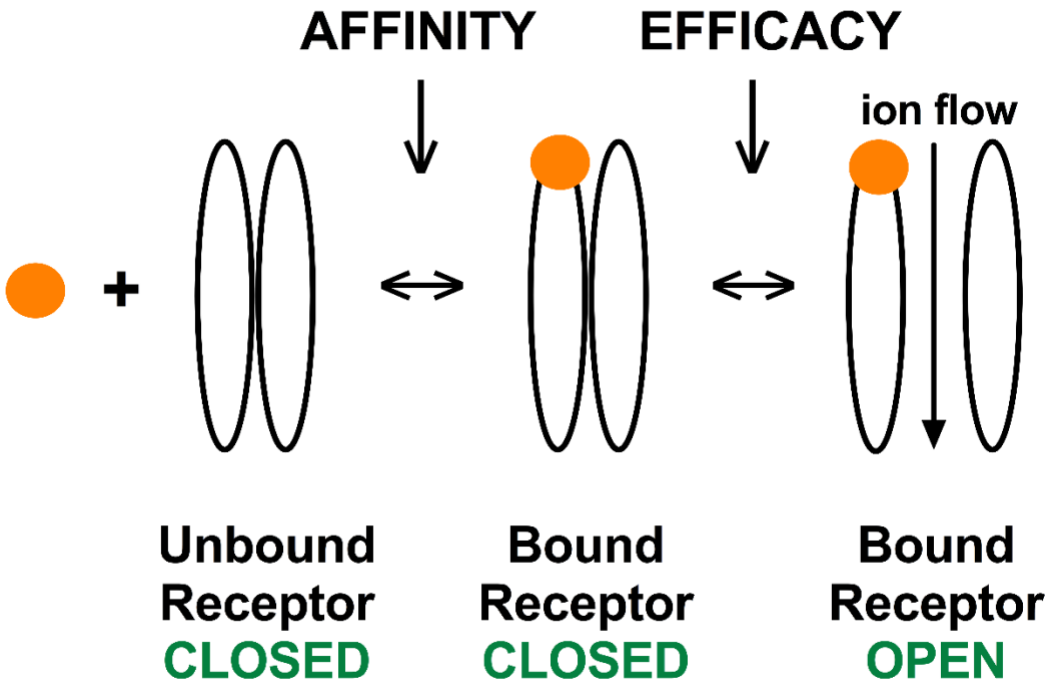

Figure 1. Simplified del Castillo-Katz mechanism of ion channel receptor activation.
A ligand (in orange) binds to an unopened receptor (left, black ovals). The ligand binds to the unopened receptor with a certain affinity for its binding site on the receptor (middle). Depending on the ligands efficacy, the ligand can produce opening of the ion channel and allow ion influx (right). The rate constant k_{+1} describes the rate of agonist binding and is described in units of $s^{-1}M^{-1}$, while k_{-1} is the rate of agonist unbinding, in units of s^{-1}. The ratio of k_{-1}/k_{+1} yields binding affinity (in molar units). The transition rate between the receptor-bound closed and open channel states of the receptor is β (expressed in units of s^{-1}) while the converse rate is α (also in units of s^{-1}) and β/α yields efficacy, which is unitless.

Prolonged activation of the receptor results in receptor desensitization, a refractory period in which the channel remains closed despite agonist being bound to the receptor (Jones and Westbrook, 1995; Jones and Westbrook, 1996). Additionally, there may be a separate step involved between agonist binding and receptor activation, known as a pre-activated flip state, at least in the cys-loop glycine and $GABA_A$ receptors. 'Flipping' the receptor from its pre-activated state results in channel activation (Burzomato et al., 2004; Lape et al., 2008; Mukhtasimova et al., 2009).

Allosteric modulators are molecules that act on a receptor through a site that is separate from the ligand binding site and are unable to activate the receptor in the absence of a ligand. Allosteric modulators, such as a variety of benzodiazepines that act at $GABA_A$ receptors, vary in their affinities for a receptor's modulatory site and exert their actions by indirectly producing a change in the affinity or efficacy of a ligand for its receptor. While modulators are often incorrectly described as having efficacy at their receptor, they have no efficacy as they are unable to activate the receptor in the absence of a receptor agonist. Receptor antagonists block agonist action by either directly competing with an agonist for its site, as is the case for a competitive antagonist, or through an action at a separate site.

1.3 Ionotropic Glutamate Receptors

Ionotropic glutamate receptors are excitatory neurotransmitter receptors that exist both pre-synaptically and post-synaptically. Activation of these receptors by a ligand results in the opening of a central cation-conducting pore, allowing for depolarization of the cell to occur. Ionotropic glutamate receptors can be split into two subtypes: N-methyl-D-aspartate (NMDA) and non-NMDA receptors. The non-NMDA receptor group consists of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors. While these receptors are all activated by

glutamate, they can be differentiated pharmacologically by synthetic compounds such as AMPA, NMDA and kainate.

Ionotropic glutamate receptors are responsible for excitatory neurotransmission in the central nervous system, with non-NMDA receptors mediating a faster activation/deactivation component of excitatory transmission than NMDA receptors (Umemiya et al., 1999). These receptors are comprised of four subunits, with each subunit made up of three transmembrane domains (TMD1, TMD3 and TMD4) and one re-entrant loop in place of TMD2 (Laube et al., 1998; Rosenmund et al., 1998). The re-entrant loop forms the pore while the ligand binding domain is formed by the N-terminal of the receptor and the extracellular loop between TMD3 and TMD4 (Kuner et al., 2001; Stern-Bach et al., 1994).

1.3.1 NON-NMDA RECEPTORS

AMPA receptors are comprised of four different subunits (GluR1, GluR2, GluR3 and GluR4) in various combinations, while kainate receptors can be comprised of five different subunits (GluR5, GluR6, GluR7, KA1 and KA2). Each subunit comprising AMPA and kainate receptors is large, with some subunits/splice variants up to almost a thousand amino acids long. Electron microscopy and X-ray crystallography have revealed that these receptors assemble as dimers of dimers, meaning two different subtypes assemble with another pair in order to form a four-subunit receptor (Safferling et al., 2001; Sobolevsky et al., 2009). The receptor is thought to be arranged so that like-subunits are diagonally opposed. While homomeric receptors do exist (meaning four identical subunits), AMPA and kainate receptors are thought to mostly exist as heterodimers.

AMPA and kainate receptors conduct Na^+ ions through their central pores once activated. However, if the AMPA receptor lacks a GluR2 subunit (which is not the case most of the time), that AMPA receptor is also permeable to Ca^{2+} ions (Sommer et al., 1991; Burnashev et al., 1996). AMPA GluR2 subunits undergo post-transcriptional Q/R RNA editing, where an uncharged glutamine (Q) at position 586 (part of the re-entrant loop) is replaced by a positively charged arginine (R). This renders the GluR2-containing AMPA receptor impermeable to Ca^{2+}. The kainate receptor subunits GluR5 and GluR6 also undergo RNA editing at this position, and thus kainate receptors containing these subunits are also not Ca^{2+} permeable. AMPA and kainate receptors that are permeable to Ca^{2+} are blocked by endogenous polyamines (eg. spermine) and display an inwardly rectifying current/voltage relationship, meaning that little current flows through these receptors at positive membrane potentials (Bowie and Mayer, 1995).

1.3.2 NMDA RECEPTORS

NMDA receptors, like AMPA receptors, are composed of four subunits, each with three transmembrane spanning domains (TMD1, 3 and 4) and with a re-entrant loop in place of TMD2, that lines the channel pore. Seven NMDA receptor subunits have been identified; NR1, NR2A, NR2B, NR2C, NR2D, NR3A and NR3B. The NR1 subunit is encoded by a single gene, but post-translational modifications can result in eight functional isoforms of this subunit. The NR2 family (NR2A, 2B, 2C and 2D) are encoded by four separate genes, and all but NR2A have splice variants. The NR3 family (A and B) has been studied less and consists of two gene members. The NR3 subunits may act as modulatory subunits in some brain regions and appear less frequently than the NR1 and NR2 subunits. The number of possible subunit combinations to form a receptor results in considerable NMDA receptor diversity. mRNA for NR1 subunits is expressed ubiquitously throughout the CNS; however, NR2 subunit expression is more varied by subtype, depending on

brain region and developmental stage (Laurie and Seeburg, 1994; Cull-Candy and Leszkiewicz, 2004).

NMDA receptors do not exist as homomers, as they require two NR1 subunits and at least one type of NR2 subunit (Monyer et al., 1992). NR1 subunits can combine with NR3 subunits in the absence of NR2, but these receptors are not responsive to glutamate, only glycine (Chatterton et al., 2002). Like the non-NMDA receptors, these receptors form heterotetramers where like-subunits are arranged diagonally opposed to one another (Salussolia et al., 2011; Riou et al., 2012; Karakas and Furukawa, 2014).

1.3.2.1 NMDA Receptor Agonist Binding and Activation

NMDA receptor activation slightly differs to that of non-NMDA receptors. Like non-NMDA receptors, NMDA receptors have an agonist binding domain formed from a clam-shell pocket created by the extracellular N-terminus and the loop between TMD3 and TMD4 (Stern-Bach et al., 1994; Furukawa and Gouaux, 2003). After agonist binding, the two domains contributing to the binding pocket close and induce a conformational change in the transmembrane domains of the receptor (Armstrong and Gouaux, 2000). While four agonists can bind to one non-NMDA receptor, this is not the case for NMDA receptors. The pocket created by NR2 subunits can bind glutamate, however the pocket created by NR1 subunits is too small for glutamate to bind (Laube et al., 1997). Instead, the pocket created by NR1 subunits (or NR3 subunits) is for glycine, a co-agonist of the NMDA receptor (Johnson and Ascher, 1987; Tikhonova et al., 2002) (**Figure 2**). D-serine is also capable of activating the glycine co-agonist site (Miller et al., 2004). NMDA receptors require both glutamate and glycine binding site occupation to open, explaining why homodimers of the NMDA receptor do not exist. While non-NMDA receptors can open with just

one glutamate molecule bound to one subunit, NMDA receptors have two agonist sites and two co-agonist sites and require that the co-agonist site is occupied before the channel can open (Clements and Westbrook, 1991; Smith and Howe, 2000).

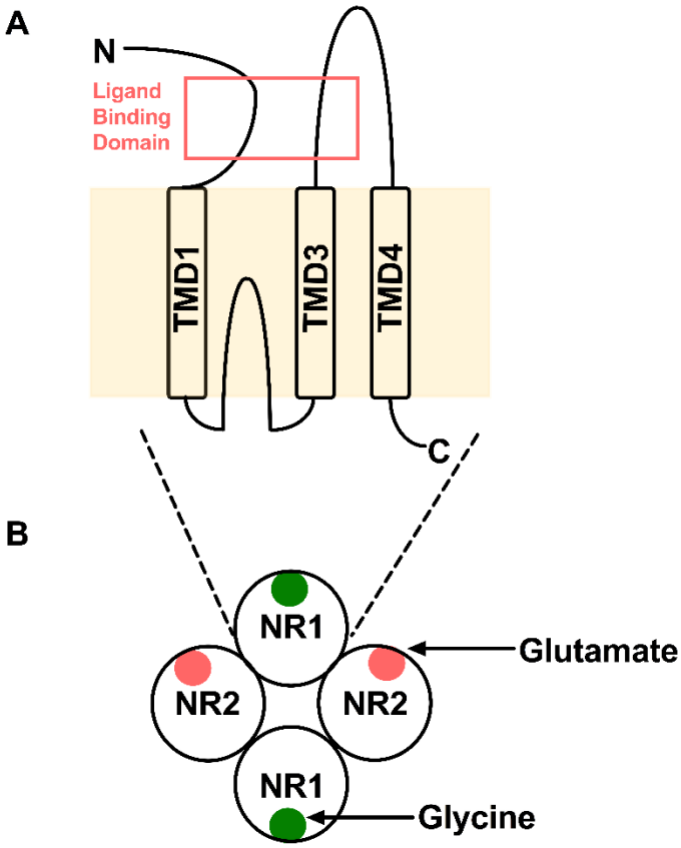

Figure 2. Illustration of the NMDA receptor.

(**A**) One ionotropic glutamate receptor subunit is comprised of three transmembrane domains and a re-entrant loop in place of transmembrane domain two. The ligand binding domain is formed by the N-terminal domain and the extracellular loop between transmembrane domains three and four. The plasma membrane is depicted in beige. Four subunits form a functional NMDA receptor as seen in (**B**), with NR1 subunits containing the co-agonist glycine binding site (in green) and the NR2 subunit containing the glutamate binding site (in pink).

In addition to the requirement of a co-agonist, NMDA receptors also have a voltage-dependent block by Mg^{2+} (Ruppersberg et al., 1994). After the cell is depolarized, the Mg^{2+} block is relieved and the channel can conduct ions through its pore. NMDA receptors are permeable to calcium ions, as they have an asparagine residue (N598) in a position homologous to that of the Q/R post transcriptional editing site in non-NMDA receptors (Burnashev et al., 1992). Mutagenesis of N598 to a charged glutamine residue in the NR1 subunit renders the NMDA receptor less permeable to Ca^{2+} and slightly reduces the Mg^{2+} block. Conversely, mutation of N598 to a charged glutamine residue in the NR2 subunit renders the NMDA receptor much less sensitive to Mg^{2+} while having almost no effect on Ca^{2+} permeability (Sakurada et al., 1993). The ability of NMDA receptor to conduct Ca^{2+} ions is thought to play a major role in synaptic plasticity (Malenka and Nicoll, 1993).

1.3.2.2 Zinc Modulation of the NMDA Receptor

Zinc is present throughout the central nervous system, with synaptic concentrations in the brain thought to reach as high as 100μM (Assaf and Chung, 1984; Frederickson, 1989). Zinc can accumulate in neurons in presynaptic vesicles via a zinc transporter and is often co-localized in brain regions with the NMDA receptor (Palmiter et al., 1996; Sindreu et al., 2003). While high concentrations of zinc can act similarly to Mg^{2+} by voltage-dependently blocking the channel pore (Kawajiri and Dingledine, 1993; Williams, 1996), it is thought to have a second site of action in the extracellular N-terminal domain region of the receptor (Choi and Lipton 1999; Fayyazuddin et al., 2000; Low et al., 2000). Binding of low concentrations of zinc to residues in the N-terminal domain of the NR2 subunit is thought to allosterically stabilize the closed state of the NMDA channel by decreasing both the probability and duration of channel opening (Erreger and Traynelis, 2008). The NR1 subunit N-terminal domain also appears to play a role, suggesting that zinc might

bind at the subunit interface between NR1 and NR2, as deletion of the NR1 N-terminal domain also eliminates zinc inhibition (Madry et al., 2007). Zinc binding to the N-terminal domain may also influence proton sensitivity of the receptor. Protons themselves act as allosteric inhibitors of the NMDA receptor, and the binding of zinc enhances the sensitivity of the receptor to proton inhibition (Choi and Lipton, 1999). This has been supported with single channel recording data showing changes in the protonation of the channels in the presence of zinc (Erreger and Traynelis, 2008). Mutagenic studies suggest that the structural rearrangement of the channel that occurs after zinc binding separates the interface between NR1 and NR2 subunits and allows proton binding to occur (Gielen et al., 2008). There are subunit differences amongst this voltage-independent modulation by zinc, with a higher sensitivity to zinc inhibition exhibited by NR2A-containing receptors (nanomolar) than NR2B-containing receptors (micromolar) (Paoletti et al., 1997; Rachline et al., 2005).

1.3.2.3 Ethanol Modulation of the NMDA Receptor

Concentrations of alcohol obtained in humans after consuming alcohol (~20-50 mM) are capable of inhibiting NMDA receptor function, as seen in brain slices and in heterologous expression systems (Lovinger et al., 1989; Lovinger et al., 1990; Chu et al., 1995; Wright et al., 1996; Xu and Woodward, 2006). Interestingly, the extent of inhibition produced by ethanol at NMDA receptors decreases quickly after a short period of time, an effect observed *in vitro* and *in vivo* and known as acute tolerance (Grover et al., 1994; Lin et al., 2003). At the single channel level, it appears that ethanol decreases the frequency of channel opening and the mean open time of the receptor (Wright et al., 1996). This effect of alcohol may be brought about by ethanol competing (directly or indirectly) with glycine at the NMDA receptor; however, other studies suggest that this is not the case (Rabe and Tabakoff, 1990; Woodward et al., 1991; Dildy-Mayfield

and Leslie, 1991; Peoples and Weight, 1992; Buller et al., 1995; Mirshashi and Woodward, 1995; Cebers et al., 1996). An amino acid (phenylalanine 639) in the TMD3 of the NR1 subunit confers sensitivity to ethanol, with mutation of this residue to alanine resulting in a decrease in receptor sensitivity to ethanol. Additionally, mutating specific residues in the NR1 subunit TMD4 enhances the receptor's sensitivity to ethanol (Ronald et al., 2001; Smothers and Woodward, 2006). Amino acids in TMD3 and TMD4 in the NR2A subunit may also play a role in conferring ethanol sensitivity (Ren et al., 2003; Honse et al., 2004; Ren et al., 2007). In contrast, the NR3 subunit is not thought to contribute towards the ethanol sensitivity of NMDA receptors (Smothers and Woodward, 2003).

1.4 Cys-Loop Receptors: $GABA_A$ and Glycine Receptors

$GABA_A$ and glycine receptors, as well as nicotinic acetylcholine and serotonergic 5-HT_{3A} receptors, belong to the superfamily of cys-loop receptors. Cys-loop receptors all have thirteen amino acids in their N-terminal domains, flanked by a cysteine on either side, which form a disulfide bond, creating a conserved ‘loop’ of amino acids. Insight into the structure of these receptors has come from electron microscopy of the *Torpedo* nicotinic acetylcholine receptor and through similarities to existing crystal structures of the acetylcholine binding protein, *Gloeobacter* ligand-gated ion channel (GLIC), *Erwinia* ligand-gated ion channel (ELIC) and glutamate-gated ion channel (GluCl) (Unwin, 1995; Brejc et al., 2001; Hilf and Dutzler, 2008; Bocquet et al., 2009; Hibbs and Gouaux, 2011). Recently, cryogenic electron microscopy solved the structure of the $GABA_A$ receptor (Zhu et al., 2018). Cys-loop receptors are comprised of five subunits, which form around a central anion or cation conducting pore. Each subunit has four transmembrane domains (TMD1-4), with a large N-terminal (containing the cys-loop), a large intracellular loop between TMD3 and TMD4, and a short C-terminal. The large N-terminal domain is where ligands

bind, and this occurs at the interface of two subunits. TMD2 primarily forms the ion-conducting pore of the channel, while the large intracellular loop is thought to be important for various receptor properties, such as receptor trafficking and post-translational modifications (Langlhofer and Villmann, 2016). The $GABA_A$ and glycine receptors have an anion-conducting pore, while nicotinic acetylcholine and serotonergic 5-HT_{3A} receptors contain a cation-conducting pore.

1.4.1 $GABA_A$ RECEPTORS

$GABA_A$ receptors, like all cys-loop receptors, are comprised of five subunits (**Figure 3**). To date, 19 subunits have been identified: six alpha (α_{1-6}), three beta (β_{1-3}), three gamma (γ_{1-3}), three pi (ρ_{1-3}) one epsilon, one omega, one delta and one pi subunit, from 16 different genes. While this would allow for enormous receptor diversity, it is estimated that under 20 combinations exist in the central nervous system (Enna, 2007). The most prevalent combination of $GABA_A$ receptor subunits appears to be the $\alpha_1\beta_2\gamma_2$ receptor, containing 2 α_1, 2 β_2 and 1 γ_2 subunit(s). $GABA_A$ receptors are expressed throughout the brain, both pre- and post-synaptically, and are thought to be the main players in regulating inhibitory transmission. Immunohistological studies in the mouse brain suggest that $\alpha_2\beta_3\gamma_2$ receptors are the second most abundant type of receptor but are more localized to certain areas compared to the $\alpha_1\beta_2\gamma_2$ receptor, including the hippocampus, striatum, olfactory bulb and the amygdala (Nusser et al., 1996; Fritschy et al., 1998; Sassoe-Pogentto et al., 2000). α_3 containing receptors are also regionally restricted to specific brain regions, including the subthalamic nucleus, paraventricular nucleus, amygdala, raphe nuclei and the basal forebrain (Gao et al., 1993; Heldt and Ressler, 2007). Receptors containing α_4, α_5, α_6 and δ subunits represent less than 10% of all $GABA_A$ receptors, are mostly extra-synaptic, and are responsible for slow tonic inhibition. The rho receptors exist as homomers or heteromers (of various ρ subunit

combinations) and are found in low levels throughout the central nervous system, but at high levels in the retina (Albecht et al., 1997; Koulen et al, 1998; Enz et al., 1999).

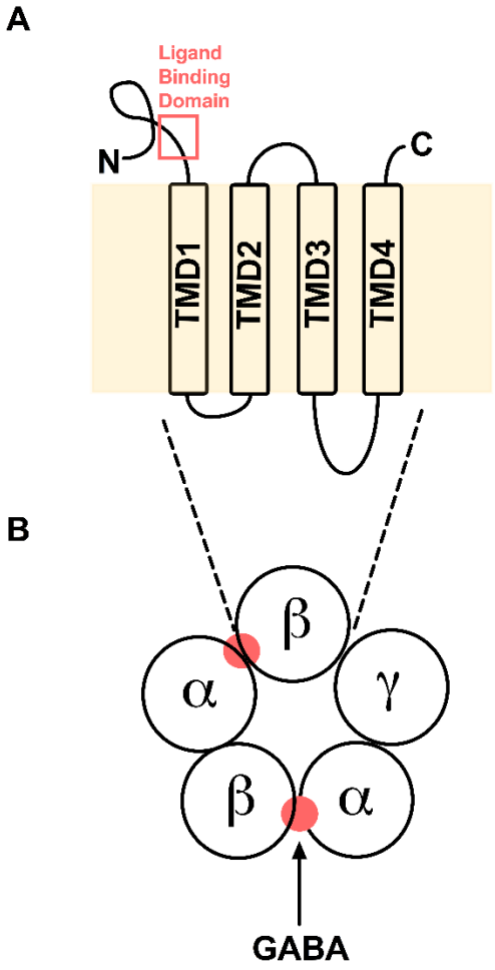

Figure 3. Illustration of the $GABA_A$ receptor.
(**A**) Each $GABA_A$ receptor subunit is comprised of four transmembrane domains, a large N-terminus which forms the agonist binding site, and a large intracellular loop between transmembrane domains three and four. The beige box represents the plasma membrane. Five subunits form a functional receptor, as seen in (**B**). The αβγ $GABA_A$ receptor contains two GABA binding sites (pink ovals), one at each α^--β^+ interface.

During development $GABA_A$ receptors, as well as glycine receptors, can produce depolarization of the cell upon receptor activation. This is because the driving force of Cl^- is reversed; after channel activation Cl^- flows out of the cell rather than into the cell, as is seen in adults. This conversion of $GABA_A$ and glycine receptors from having excitatory to inhibitory actions is thought to be due to an increase in expression of the K^+/Cl^- KCC2 transporter during development (Rivera et al., 1999; Hubner et al., 2001).

The $GABA_A$ receptor agonist binding site is at the $\alpha^-\beta^+$ interface, explaining the need for both α and β subunits to form a functional receptor ($GABA_A$ rho receptors are the only exception) (Smith and Olsen, 1995). Tyrosine 97 and leucine 99 are thought to line the GABA binding site on the β subunit side, and leucine 99 of the β subunit may play a role in coupling GABA site agonist binding to channel opening (Boileau et al., 2002; Laha and Tran 2012). Upon activation by its ligand (e.g. GABA), $GABA_A$ receptors undergo a conformational change which results in channel opening, allowing Cl^- to flow into the cell, ultimately resulting in cell hyperpolarization. It is unclear exactly how receptor binding triggers channel opening, but the conformational wave that occurs during this process may involve a series of electrostatic interactions between charged amino acids, which are broken or formed after agonist binding. Several studies have implicated electrostatic interactions within or between the α and β subunits before or after GABA binding. In the N-terminal region, the positively-charged arginine 120 of the α_1 subunit may form an interaction with the negatively-charged aspartic acid 153 of the β_2 subunit after GABA binds (Laha and Wagner, 2011). An electrostatic interaction between lysine 279, found in the α_1 linker region between TMD2 and TMD3, with either aspartic acids 149 or 57, both found in the α_1 N-terminal region, may be key to coupling agonist binding to channel opening (Kash et al., 2003). Another study found that glutamic acid 153 and lysine 196 in the N-terminal within the same β_2 subunit interact to stabilize the open state of the channel (Venkatachalan and Czajkowski, 2008). The

involvement of electrostatic interactions in the coupling of agonist binding to channel gating is not unique for $GABA_A$ receptors, as similar electrostatic interactions have also been implicated in other cys-loop receptors (Mukhtasimova et al., 2005; Todorovic et al., 2010).

1.4.1.1 Benzodiazepine Modulation of the $GABA_A$ Receptor

Benzodiazepines have been widely prescribed since the 1960s. Drugs belonging to this class have the structure of a benzene ring fused with a diazepine ring, and include Valium® (diazepam) and Xanax® (alprazolam). These compounds are used therapeutically for their anti-insomnia, anxiolytic and anti-convulsant properties. Unfortunately, tolerance to this class of compounds develops quickly, and their reinforcing properties make them a bad long-term treatment option. Benzodiazepines act at the $\alpha^-\text{-}\gamma^+$ N-terminal domain interface of the $GABA_A$ receptor. Due to this site of action, benzodiazepines have effects only on $GABA_A$ receptors containing both an α and a γ subunit. Benzodiazepines act as positive modulators of the $GABA_A$ receptors, meaning that, when applied alone, they exert no effect. Once GABA is bound, they can potentiate the $GABA_A$ receptor. Several mechanisms as to how this enhancement is produced have been described, including benzodiazepines decreasing the rates of GABA unbinding, increasing GABA binding rates, increasing single channel conductance and enhancing an intermediate pre-activation step that occurs after GABA binding, but before channel opening (Twyman et al., 1989; Rogers et al., 1994; Eghbali et al., 1997; Campo-Soria et al., 2006; Bianchi et al., 2009; Gielen et al., 2012). Despite many hypotheses, there is still much unknown about how benzodiazepine binding is coupled to enhancement of receptor function.

Receptors that consist of $\alpha_{1\text{-}3,5}$ in combination with $\gamma_{1\text{-}3}$ are sensitive to modulation by benzodiazepines, with γ_1 and γ_3 having reduced sensitivity to benzodiazepines compared to γ_2

subunit-containing receptors (Ymer et al., 1990; Herb et al., 1992). The histidine 101 residue in the α_1 subunit plays an important role in benzodiazepine action and it is conserved in the $\alpha_{2,3,5}$ subunits (Wieland et al.,1992; Benson et al., 1998). Receptors that contain the α_4 or α_6 subunit are insensitive to benzodiazepines as they have an arginine residue in place of the conserved histidine residue. Mutations of α subunits at the conserved histidine residue renders those receptors insensitive to benzodiazepine modulation. Knock-in mice expressing these benzodiazepine-insensitive mutations in different subunits have demonstrated that specific $GABA_A$ receptor subunits mediate specific behavioral effects of benzodiazepines. The α_1(H101R) mutant mouse, in which amino acid residue 101 has been mutated from histidine to arginine, is resistant to the sedating effects of benzodiazepines (Rudolph et al., 1999; McKernan et al., 2000). In contrast, the α_2 (H101R) mutant mouse is resistant to the anxiolytic effects of benzodiazepines (Löw et al., 2000; Morris et al., 2006). Additionally, mice that lack the α_2-containing $GABA_A$ receptor, or have mutations that render their $\alpha_2\beta_3\gamma_2$ $GABA_A$ receptor benzodiazepine-insensitive, show diminished anti-hyperalgesic effects in response to benzodiazepines, indicating a possible therapeutic role of benzodiazepines for hyperalgesia (Paul et al., 2014).

The literature is conflicted over which α subtype mediates the 'addictive' properties of benzodiazepines. $\alpha_{1,2,3}$ and α_5 containing $GABA_A$ receptors have all been implicated. Drug addiction involves complex changes in brain circuitry, gene expression and receptor function, and an important aspect believed to be involved in this process is the increase in dopamine signaling from the ventral tegmental area to the nucleus accumbens (Gonzales et al., 2004). GABAergic neurons that project from the nucleus accumbens to the ventral tegmental area normally inhibit these dopaminergic neurons from firing. Drugs of abuse that act on the $GABA_A$ receptor (in addition to opioids and cannibinoids) are thought to produce a disinhibition of dopamine neurons in the ventral tegmental area, thereby increasing the release of dopamine into the nucleus

accumbens (Kalivas et al., 1990; Johnson and North, 1992; Szabo et al., 2002; Tan et al., 2010). α_1(H101R) knock-in mice do not develop preference for benzodiazepines, and ventral tegmental area brain slice recordings show that this mutation in the α_1 subunit prevents benzodiazepines from disinhibiting dopamine firing (Tan et al., 2010). However, a more recent study found that stimulation of both α_1- and α_2- containing $GABA_A$ receptors in the nucleus accumbens may contribute to the reinforcing properties of benzodiazepines (Engin et al., 2014; Ralvenius et al., 2015). Another study found that α_2 and α_3, but not α_1, subunits are responsible for the rewarding effects of benzodiazepines (Reynolds et al., 2012). Yet other studies have suggested that the α_3-containing $GABA_A$ receptors are primarily responsible for the rewarding effects of BZDs (Shinday et al., 2013). Tolerance to the sedative effects of diazepam has been linked to benzodiazepine activity at both α_1 and α_5- containing $GABA_A$ receptors (van Rijnsoever et al., 2004). Perhaps these conflicting results suggest that the reinforcing effects of BZDs may require simultaneous stimulation of multiple subtype compositions of the $GABA_A$ receptors, and so selectively activating only one subtype of $GABA_A$ receptor may bypass the rewarding properties of benzodiazepines.

Benzodiazepine-site acting drugs that do not produce pronounced potentiation of the $GABA_A$ receptor have also been identified. Ro 15-4513 produces a weak inhibitory effect at the $GABA_A$ receptor when added in combination with GABA, while flumazenil is termed a benzodiazepine-site antagonist. Since it is an anxiogenic compound, Ro 15-4513 is used for experimental purposes only, but flumazenil is used clinically to treat benzodiazepine overdose (Sivilotti, 2016). Flumazenil produces a very weak enhancement at the receptor when combined with GABA, but application of this compound will compete with a potentiating benzodiazepine, resulting in a decrease in the net amount of potentiation of $GABA_A$ receptor function (Mihic et al., 1994).

1.4.1.2 Zinc Modulation of the $GABA_A$ receptor

Zinc is a negative modulator of the $GABA_A$ receptor. Its degree of inhibition is subtype specific, with αβ receptors exhibiting greater sensitivity to zinc (low nanomolar) than αβγ receptors (high micromolar) (Draguhn et al., 1990; Smart et al., 1991). There are thought to be three distinct sites at the αβ $GABA_A$ receptor to which Zn^{2+} can bind, two at the extracellular αβ interface, and one within the channel pore of the β subunit. α_1 subunit residues glutamic acid 137 and histidine 141 coordinate with β subunit glutamic acid 182 to form two identical sites at two separate αβ interfaces. β subunit residues histidine 267 and glutamic acid 270 contribute to the third binding site within the pore. Mutation of all these residues (at once) to alanine results in a receptor that is insensitive to the inhibitory effects of zinc even at a concentration of 10mM Zn^{2+} (Horenstein and Akabas, 1998; Hosie et al., 2003).

Receptors that incorporate the γ_2 subunit are less sensitive to Zn^{2+}, but mutating γ subunit residues (arginine 197, isoleucine 282 and lysine 285) to mimic the histidine and glutamic acid residues in the β subunit thought to contribute to zinc binding (glutamic acid 182, histidine 267, glutamic acid 270) partially restores sensitivity to low zinc concentrations (Hosie et al., 2003). The γ_2 subunit-containing $GABA_A$ receptors are thought to contain two separate Zn^{2+} binding domains within the γ_2 subunit - one in the extracellular N-terminal domain and one near the channel pore (Nagaya and Macdonald, 2001). Delta- or epsilon-containing GABA receptors appear to be less sensitive to Zn^{2+} than αβ receptors, but more sensitive than αβγ receptors (Whiting et al., 1997; Kaur et al., 2009).

1.4.1.3 Neurosteroid Modulation of the $GABA_A$ receptors

Several progesterone and deoxycorticosterone metabolites, known as neurosteroids, are capable of positively or negatively modifying the $GABA_A$ receptor. Neurosteroids that increase $GABA_A$ receptor function can act as anaesthetics and have therapeutic potential for the treatment of insomnia, epilepsy and anxiety (Zorumski et al., 2000). The neurosteroids 5a-pregnan-3a-ol-20-one (allopregnanolone) and 3α,21-dihydroxy-5α-pregnan-20-one (THDOC) are endogenous positive modulators of $GABA_A$ receptors (Wang, 2011). Low concentrations of neurosteroids are capable of allosteric modulation of the receptor, while high concentrations directly activate the receptor through a separate binding site. Modulation of the receptor by neurosteroids seems to be dependent on several residues located in TMD1 and TMD4 within the same α subunit, while direct activation of the $GABA_A$ receptor by neurosteroids is thought to occur after neurosteroid binding to a site formed by TMD2 of the α subunit and TMD2 of the β subunit (Hosie et al., 2006; Akk et al., 2008; Hosie et al.,2008; Bracamontes et al., 2012). However, more recent crystal structures of chimeric receptors with bound THDOC have suggested that the modulatory site of neurosteroids is also formed by an intra-subunit pocket, with TMD1 from one subunit coordinating with TMD2 of the adjacent subunit (Laverty et al., 2017; Miller et al., 2017). The presence of the δ subunit versus the γ does not change receptor potentiation by neurosteroids, further suggesting that neurosteroid modulation and activation only requires the α and β subunits (Hosie et al., 2008).

Neurosteroids, such as pregnanolone sulfate, that induce an inhibitory allosteric response in the $GABA_A$ receptor, are thought to bind to a separate site from the positive allosteric neurosteroid site (Akk et al., 2001). Crystal structures of $GABA_A$ receptor chimeras suggests that pregnanolone sulfate binds perpendicularly to the membrane in a pocket formed by TMD3 and TMD4 within the same α subunit (Laverty et al., 2017). While studies have proposed that inhibitory neurosteroids may act to increase receptor desensitization, the mechanism of how

inhibitory neurosteroid binding affects channel function is still unclear (Shen et al., 2000; Akk et al., 2001).

1.4.1.4 Ethanol Modulation of the $GABA_A$ Receptor

While ethanol acts as a negative allosteric modulator of excitatory NMDA receptor function, it acts as a positive allosteric modulator of the αβ and αβγ $GABA_A$ receptor (Nakahiro et al., 1991; Reynolds et al., 1992; Tatebayashi et al., 1998). Two amino acids in the α_1 subunit TMD2 (serine 270) and TMD3 (alanine 291), and one in the β_1 subunit TMD2 (serine 265) influence ethanol modulation of the $GABA_A$ receptor. α_1 residue serine 267 plays the most direct role in alcohol binding and determines how large of an alcohol molecule can bind (Mihic et al., 1997; Ueno et al., 1999; Mascia et al., 2000; Jung and Harris, 2006). Apart from the potentiating site, short-chain alcohols can also bind to an inhibitory site of the $GABA_A$ receptor, with the net effect of these short chain alcohols determined by their actions at both sites. Threonine 261 in the α_2 subunit TMD2 may mediate the inhibitory action of alcohols, with mutation of this site to tryptophan resulting in greater enhancement of receptor function by small-chain alcohols (Ueno et al, 2000; Johnson et al., 2012). On the single channel level, the addition of alcohol results in an increase in frequency of channel opening, the mean open time, burst frequency and burst duration, while decreasing the amount of time the receptor spends in the closed state (Tatebayashi et al., 1998).

Different receptor subtypes vary in their responses to ethanol, with the most prevalent $GABA_A$ receptor, $\alpha_1\beta_2\gamma_2$ receptors, exhibiting low sensitivity to ethanol. αβδ receptors have been proposed as the "one drink target" for low concentrations of ethanol, but the alcohol sensitivity of these receptors is debated. While some studies suggest αβδ receptors are enhanced by low

concentrations (1-30mM) of ethanol, others have failed to see this effect (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Borghese et al., 2006; Yamashita et al., 2006; Baur et al., 2009). Mutagenesis studies have pointed at the alcohol binding site in αβδ receptors to be at the N-terminal α^+/β^- interface-the interface that does not bind GABA (Wallner et al., 2014). Further, these low ethanol effects on αβδ receptors may be antagonized by Ro 15-4513, the benzodiazepine-site inverse agonist, which is also hypothesized to have a site of action on benzodiazepine-insensitive receptors (Hanchar 2006; Wallner et al., 2006; Wallner et al., 2014).

The GABA rho receptor lacks the potentiating alcohol-binding site (it has an isoleucine residue in place of serine 270) present in αβ and αβγ receptors and thus alcohols inhibit rho receptor function. A threonine residue (T298) in TMD2 of the rho subunit is thought to be key in producing this inhibitory response. (Wick et al., 1998; Borghese et al., 2015).

1.4.2 GLYCINE RECEPTORS

Glycine, while acting as a co-agonist for excitatory NMDA receptors, can also act as an agonist at inhibitory glycine receptors. These receptors belong to the cys-loop family of receptors, are anion conducting, and mediate inhibitory synaptic transmission. These receptors are composed of α subunits, and are sometimes expressed with a β subunit. To date, four different α subunits one β subunit have been identified. While each α subunit arises from a separate gene product, the α subunits share over 90% sequence identity. Glycine receptors, while originally thought to be localized to the spinal cord, are expressed throughout the central nervous system, including in the nucleus accumbens, hippocampus, and in thalamic and brainstem nuclei (Zarbin et al., 1981; Colin et al., 1998; Ghavanini et al., 2005; Molander and Soderpalm, 2005).

Glycine receptors can exist as homomers, with five identical copies of the same α subunit, as α subunit heteromers, or as αβ heteromers. β homomers are not believed to form functional receptors (Bormann et al., 1993). The glycine receptor agonist site is at the interface of two adjacent subunits, resulting in a glycine receptor having five possible binding sites for glycine. The receptor, as with all cys-loop receptors, is thought to follow a concerted model of opening, where a single molecule binding to an agonist site results in all subunits undergoing simultaneous conformational changes, resulting in channel opening (Corringer et al., 2000). Gating efficacy increases as more glycine agonists bind to the remaining four sites, with maximal efficacy achieved when only three of the five sites are occupied (Beato et al., 2004). There is little difference in glycine sensitivity between subunits (Lynch, 2004).

1.4.2.1 Zinc Modulation of the Glycine Receptor

Zinc acts as a biphasic modulator of the glycine receptor. At low (nanomolar) concentrations, zinc potentiates glycine receptor responses, while high concentrations (>10 μM) inhibit the receptor. This is thought to be due to the presence of two distinct zinc binding sites on the glycine receptor. Several residues have been implicated in the potentiating effects of zinc. Mutagenesis of aspartic acid 80 of the α_1 subunit disrupts zinc enhancement of glycine-activated currents, but not taurine-activated currents, complicating this residue's involvement in zinc binding to its high affinity potentiating site (Laube et al., 2000). Further mutagenic studies have found that alanine substitution of histidine 215 significantly reduces zinc potentiation of receptor function, while alanine substitution of glutamic acid 192 and aspartic acid 194 eliminates the enhancing action by zinc (Miller et al., 2005). Interestingly, serine substitution of tryptophan 170 also eliminates zinc potentiation, and this is thought to be due to disruption of the zinc binding site formed by the previously mentioned residues (Zhou et al., 2013; Cornelison et al., 2017). The low

affinity zinc inhibition site is thought to bind zinc via the coordination of two histidine residues (H107, H109) at the N-terminal interface of two adjacent subunits. Mutation of these histidine residues eliminates the inhibitory effects seen at high zinc concentrations (Harvey et al., 1999; Nevin et al., 2003).

1.4.2.2 Ethanol Modulation of the Glycine Receptor

Ethanol potentiates glycine receptor function, and the receptor is more sensitive to lower concentrations of ethanol than the $\alpha\beta\gamma$ $GABA_A$ receptor. On the single channel level, ethanol acts by increasing channel burst duration by reducing the rate of glycine unbinding from the receptor (Welsh et al., 2009). Through chimeric constructs and mutagenesis, amino acids in the α_1 subunit TMD2 (serine 267) and TMD3 (alanine 288) were found to play an important role in ethanol modulation of the receptor (Mihic et al., 1997). The residue present at position 267 in the α_1 subunit can determine the extent of potentiation produced in the receptor by ethanol. A large residue in position 267 results in ethanol allosterically inhibiting the receptor, while small residues (like the serine present in wildtype receptors) allow ethanol to potentiate the receptor (Ye et al., 1998).

1.5 Targeting $GABA_A$, Glycine and NMDA Receptors for the Treatment of Disease

NMDA, $GABA_A$ and glycine receptors play a major role in the excitatory and inhibitory transmission in the brain, thus it is not surprising that they have therapeutic potential for a large range of indications. These indications include, but are not limited to, anxiety, depression, drug abuse, epilepsy, pain, Alzheimer's disease, Parkinson's disease, schizophrenia and stroke.

1.5.1 Anxiety and Depression

Ketamine, which acts as an open channel blocker of the NMDA receptor, has recently shown promise as a fast-acting antidepressant (Berman et al., 2000; DiazGranados et al., 2010). While it is unclear how ketamine is producing its anti-depressant effects, it has brought attention to the NMDA receptor for its therapeutic potential in treating depression (Zanos and Gould, 2018). Antagonists at the glycine co-agonist site (e.g. AV-101) have also generated interest for their anti-depressant effects (Przegalinski et al., 1997; Duman, 2018).

Ligands selective for α_1- containing $GABA_A$ receptors (e.g.zolpidem) are used clinically to treat insomnia, while the α_2 containing $GABA_A$ receptor is a target for non-sedating anxiolytics (Löw et al., 2000; McKernan et al., 2000; Morris et al., 2006). However, drugs (eg. TPA-023, a partial agonist at α_2/α_3 $GABA_A$ receptors) targeting the α_2 subtype have failed to make it through the clinical trial process (Atack, 2008; Ator et al., 2010; Mohler, 2011). Activation of the glycine receptor with taurine produces an anxiolytic response, suggesting a possible role of glycine receptors in mediating anxiety responses (Zhang and Kim, 2007).

1.5.2 Drug Abuse

NMDA receptor modulators have been implicated in the treatment of drug addiction and alcoholism (Tomek et al., 2013). Excitatory synapses on ventral tegmental dopaminergic neurons are strengthened by drugs of abuse, through the cell membrane insertion of GluR2-lacking (Ca^{2+} permeable) AMPA receptors. NMDA receptor antagonists can block this strengthening from occurring (Ungless et al., 2001; Saal et al., 2003). Additionally, the NMDA receptor negative modulator (uncompetitive antagonist) memantine has shown clinical promise in reducing cue-induced craving in alcoholics (Krupitsky et al., 2007).

Potentiators and inhibitors of $GABA_A$ receptor function also show clinical promise for aspects of drug use and dependence. While benzodiazepines are often clinically used to ease patients out of alcohol withdrawal, the abuse liability associated with benzodiazepines themselves leaves a lot to be desired as a drug withdrawal antidote (Liang and Olsen, 2014). However, the effect of $GABA_A$ receptor potentiation on alcohol withdrawal validates the receptor as a target for treatment of drug abuse. This is supported by the actions of flumazenil, which is used to counteract benzodiazepine overdose, and the inverse benzodiazepine-site agonist Ro 15-4513, which antagonizes the behavioral effects of alcohol, and reduces alcohol consumption in rodents (Suzdak et al., 1988; June et al., 1991).

Glycine receptor acting drugs may also have therapeutic potential for drug abuse. The increase in dopamine transmission into the nucleus accumbens after alcohol consumption in rodents is dependent on glycine receptor activation, with a glycine receptor antagonist preventing this increase (Molader and Soderpalm, 2005; Jonsson et al., 2014). Additionally, there is evidence that pharmacological activation of glycine receptors (essentially mimicking alcohol potentiation

of glycine receptors) in the ventral tegmental area or nucleus accumbens decreases alcohol consumption (Molander et al., 2005; Li et al, 2012).

1.5.3 PAIN

NMDA, $GABA_A$ and glycine receptors are all implicated in pain pathways. The α_3 glycine receptor is highly expressed in the spinal cord dorsal horn and is inhibited by prostaglandin E2's activation of protein kinase A. Protein kinase A activation leads to internalization of the α_3 receptor and decreases receptor function (Ahmadi et al., 2002; Harvey et al., 2004). Decreased α_3 receptor number and function results in an increase in nociceptive pain signaling to the brain; therefore, increasing glycine receptor function may be of therapeutic interest for the treatment of pain.

Additionally, glutamate-induced neurodegeneration of GABAergic neurons in the dorsal horn may play a role neuropathic pain, with selective deletion of NMDA receptors in the dorsal horn decreasing the amount of neurodegeneration and pain observed in rodents (Inquimbert et al., 2018). Additionally, numerous other studies have shown that blocking NMDA receptors can relieve pain in rodent models (Davar et al., 1991; Ren et al., 1992; Mao et al., 1993). Enhancing the lost GABAergic transmission in the dorsal horn may also relieve pain by re-establishing the fine balance between excitation and inhibition (Koga et al., 2017).

1.5.2 OTHER DISORDERS

Epilepsy can result from the imbalance between excitation and inhibition in the brain (i.e. too much excitation and/or too little inhibition). Thus it is unsurprising, given the NMDA receptor's role in excitatory transmission, that a reduction in NMDA transmission may be

beneficial in the treatment of epilepsy (Urbanska et al., 1998). Felbamate, which may in part exert its actions via NMDA receptor blockade, has anti-convulsant effects (Subramaniam et al., 1995). Despite multiple animal studies promising anti-convulsant effects from other inhibitors of NMDA receptor function (e.g. dizocilpine, a non-competitive NMDA receptor antagonist), contradictory results and undesirable side effects in animal studies have thus far hindered their progress to the clinic (Wong et al., 1986; Sato et al., 1988; Ormandy et al., 1989; Wozniak et al., 1996; Ghasemi and Schachter, 2011).

The role of $GABA_A$ receptors in epilepsy is evidenced by mutant receptors (such as the K289M mutation in the γ_2 subunit), where abnormal GABA pharmacology is associated with epileptic seizures (Baulac et al., 2001; Audenaert et al., 2006). Barbiturates, positive allosteric modulators of the $GABA_A$ receptor, were the first drugs to be used in the treatment of epilepsy. Benzodiazepines remain first in line for status epilepticus and are sometimes prescribed for prophylactic purposes, but their abuse potential and sedative effects calls for the use of other agents and the development of $GABA_A$ receptor subunit selective anti-epileptic drugs (Ochoa et al., 2016). Similarly, RNA editing of α_2 and α_3 glycine receptors results in receptors with enhanced receptor function, and is associated with temporal lobe epilepsy (Meier et al., 2005; Eichler et al., 2008; Eichler et al., 2009). Selectively blocking these receptors would be therapeutically beneficial.

An over-activation of NMDA receptors, leading to an excess of Ca^{2+} influx and excitotoxicity-mediated cell death, is believed to contribute to other neurological disorders besides epilepsy, such as Alzheimer's disease, Parkinson's disease and stroke (Mody and Macdonald, 1995; Bleich et al., 2003). While NMDA receptor modulators have shown therapeutic potential for these indications in animal models, only memantine (a negative NMDA receptor modulator

used to treat Alzheimer's) has made it to the clinic (Hallett and Standaert, 2004; Hoyte et al., 2004; Olivares et al., 2012). This setback may be due to a lack of specificity of antagonists studied, resulting in unwanted side effects, and has resulted in a shift for pharmaceutical companies to look towards more specific NMDA receptor modulators, or to instead target pathways downstream from NMDA receptors (Olney et al., 1989; Vandame et al., 2013; Wu et al., 2018).

In this dissertation, I investigated several components of allosteric modulation. First, I identified the endogenous ketone bodies acetone and β-hydroxybutyric acid as allosteric modulators of the NMDA, $GABA_A$ and glycine receptor. This allosteric modulation may have therapeutic benefit for the various indications mentioned above. Second, I investigated the molecular mechanism of benzodiazepine action with the goal of gaining insight towards how to achieve drug selectivity. Lastly, I utilized phage display technology to identify peptides capable of modulating the α_2 $GABA_A$ receptor, in hopes of finding a non-sedative anxiolytic that may also have other therapeutic indications.

CHAPTER 2: KETONE BODY MODULATION OF LIGAND-GATED ION CHANNELS[1]

2.1 Introduction

Ketogenesis, the production of ketone bodies from fatty acids, occurs constantly at low rates in the human body. Under certain conditions, however, such as in situations of low blood glucose, ketogenesis becomes the body's main energy resource. Fatty acids are broken down in liver mitochondria into acetyl-CoA molecules, which at high levels can overwhelm the citric acid cycle. Acetyl-CoA is then instead further metabolized in a series of steps into the ketone body acetoacetate, which itself breaks down into the ketone bodies acetone and β-hydroxybutyrate. While β-hydroxybutyrate is not technically a ketone, as its carbonyl side group is only attached to one carbon not two, it is frequently referred to as a ketone body. Acetone is a small polar molecule that readily diffuses away from the liver to be metabolized further into D-lactate, pyruvate and glucose (Glew, 2010). The liver is unable to use acetoacetate and β-hydroxybutyrate as metabolic fuel due to a lack of succinyl CoA transferase (Williamson et al., 1971; Orii et al., 2008), an enzyme necessary for the re-conversion of acetoacetate and β-hydroxybutyrate back to acetyl-CoA. Instead, acetoacetate and β-hydroxybutyrate diffuse away from the liver to other regions, and can even pass into the brain via monocarboxylate transporters (Leino et al., 2001; Kim & Rho, 2008).

Excessive production of ketone bodies can result in ketoacidosis, in which the blood pH dips to dangerously low levels. Risk factors for ketoacidosis include type I diabetes and heavy alcohol consumption, although it is not clear if acidosis is produced by alcohol consumption

[1] This chapter details work that has been accepted for publication. Pflanz N.C. was responsible for the design, conduction and analysis of these experiments and for writing the manuscript.
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directly, or perhaps indirectly by other factors such as poor nutrition seen in individuals who consume high quantities of alcohol (Lefevre et al., 1970; Cooperman et al., 1974; Fulop & Hoberman, 1979; Derr et al., 1983; Wrenn et al., 1991; Sarkola et al., 2002; Misra & Oliver, 2015;). Despite negative consequences arising from high levels of ketone bodies, the ketogenic diet, involving low carbohydrate and high fat intake, has been used to effectively control epileptic seizures since the 1920s (Wilder, 1921). Development of anti-seizure compounds decreased the use of the ketogenic diet as a therapeutic tool but its use resurfaced in the 1990s after multiple studies demonstrated its efficacy (Freeman et al., 1998; Vining et al., 1998). Since then the ketogenic diet has been therapeutically linked to the amelioration of other disease states, including alcohol withdrawal syndrome, Parkinson's disease, amyotrophic lateral sclerosis, schizophrenia and Alzheimer's disease (Reger et al., 2004; Vanitallie et al., 2005; Siva, 2006; Kraft & Westman, 2009; Yang & Cheng, 2010; Shaafi et al., 2016; Dencker et al., 2018; Taylor et al., 2018).

Despite the various clinical indications for which a ketogenic diet may be used, its mechanisms of action remain to be elucidated. Multiple hypotheses have been suggested, including enhancement of GABAergic transmission, antagonism of glutamatergic transmission, increases in potassium channel activity, purinergic transmission or BDNF expression, changes in metabolic factors, increased antioxidant effects or an increase in, and subsequent actions of, polysaturated fatty acids (De Vivo et al., 1978; Erecińska et al., 1996; Yudkoff et al.,1997; Yudkoff et al., 2001; Deransart et al., 2003; Fraser et al., 2003; Wang et al., 2003; Dahlin et al., 2005; Bough et al., 2006; Ma et al., 2007; Maalouf et al., 2007; Yang et al., 2007; Juge et al., 2010; Masino et al., 2011; Tanner et al., 2011; Sleiman et al., 2016).

The beneficial effects of a ketogenic diet to treat both epilepsy and alcohol withdrawal syndrome led us to hypothesize that ketone bodies would have direct effects on the receptors implicated in these disorders. This chapter reports on the modulatory effects of β-

hydroxybutyrate and acetone on neurotransmitter-activated NMDA, $GABA_A$ and glycine receptors.

2.2 Methods

2.2.1 Reagents

All reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated.

2.2.2 DNA preparation & site-directed mutagenesis

Human cDNAs encoding the α_1, β_2 and γ_2 $GABA_AR$ subunits, the α_1 glycine receptor subunit (subcloned into the pBK-CMV vector), and NR1, NR2A and NR2B NMDA receptor subunits (subcloned into the pCDNA1 vector) were used in this study. The α_1, β_2 and γ_2 $GABA_AR$ subunit DNAs were injected into oocytes at a concentration of 1.5ng/30nl in a 1:1:10 ratio to ensure incorporation of the γ_2 subunit. The α_1 glycine receptor DNA was also injected at this concentration. Point mutations were introduced into the α_1 glycine and the NR1 NMDA receptor subunits using a QuikChange site-directed mutagenesis kit (Agilent Technologies, CA). Double-stranded DNA sequencing confirmed the introduction of these mutations.

2.2.3 Preparation of cRNA

Human cDNAs encoding the NR1, $NR1_{F639A}$, NR2A and NR2B genes were linearized with the restriction enzymes XbaI (NR1, $NR1_{F639A}$) or XhoI (NR2A, NR2B) (New England Biolabs, MA) and transcribed into cRNA using the mMessage mMachine™ T7 transcription kit (Thermo

Fisher Scientific, MA). NR1 was mixed with either NR2A or NR2B, and $NR1_{F639A}$ was mixed with NR2A, in a 1:1 ratio and 1.5ng/30nl of each cRNA mixture was injected into each oocyte.

2.2.4 COLLECTION AND PREPARATION OF XENOPUS LAEVIS OOCYTES

Xenopus laevis (Nasco, WI) were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited facility in a room kept at 17°C and a 12h light/dark cycle, in tanks monitored for water pH and conductivity. Oocytes were surgically removed in accordance with the National Institute of Health guidelines under a protocol approved by the IACUC of the University of Texas at Austin, and placed in a hypertonic solution (108 mM NaCl, 1 mM EDTA, 2 mM KCl, and 10 mM HEPES). The thecal and epithelial layers of Stage V and VI oocytes were manually removed using forceps. Isolated oocytes were transferred to a solution (83 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$ and 5 mM HEPES) containing 0.5 mg/mL collagenase from Clostridium histolyticum for 10 minutes in order to enzymatically remove the follicular layer of the oocytes. The animal poles of oocytes were then injected using a Nanoject II (Drummond Scientific Co., PA) with 1.5 ng/ 30nL of human α_1, β_2 and γ_2 $GABA_A$ receptor subunit cDNAs in a 1:1:10 ratio. cRNA was injected into the oocyte cytoplasm at the interface of the animal and vegetal poles. Oocytes were stored singly in 96-well plates containing incubation media (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM $MgSO_4 \cdot 7H_2O$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, 2mM sodium pyruvate, 0.5 mM theophylline, 10 U/L penicillin, 10 mg/L streptomycin). The oocytes were kept at room temperature (20°C) and away from light.

2.2.5 TWO-ELECTRODE VOLTAGE CLAMP ELECTROPHYSIOLOGY

Electrophysiological recordings were performed on oocytes 1-3 days post-injection. Individual oocytes were placed in a 100μL bath and continuously perfused at a rate of 2ml/min with buffer, which differed depending on the receptor under study.

Oocytes expressing $GABA_A$ receptors were perfused with ND-96 (96mM *NaCl*, 2mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 5mM HEPES, pH 7.4), while oocytes expressing glycine receptors were perfused with modified Barth's solution (88mM NaCl, 1mM KCl, 2.4mM $NaHCO_3$, 10mM HEPES, 0.82mM, $MgSO_4 \cdot 7H_2O$, 0.33mM Ca(NO3)2, and 0.91mM $CaCl_2$, pH 7.5). NMDA receptor-expressing oocytes were perfused with Ca^{2+} free frog Ringer's solution (115mM NaCl, 2.5mM KCl, 10mM HEPES, 1.8mM $BaCl_2$, pH 7.2).

Animal poles of oocytes were impaled with two 3M KCl-filled borosilicate glass electrodes, each with a resistance between 0.5 and 10MΩ. Oocytes were voltage clamped at -70mV ($GABA_A$ receptor expressing oocytes were clamped at -80mV) using an OC-725 oocyte clamp (Warner Instruments, CT) and data was collected at a rate of 1 kHz using a digitizer (PowerLab ML866) and LabChart (v. 7.4.7) software (both from ADInstruments, Australia).

2.2.6 TESTING RECEPTOR MODULATION BY ZINC, ETHANOL, ACETONE AND β-HYDROXYBUTYRIC ACID

An oocyte was exposed to a maximally-effective concentration of agonist (GABA for $GABA_AR$, glycine for glycine receptor, L-glutamic acid + 10μM glycine for NMDAR) applied for 30 seconds. After this, a concentration which produced 5-10% of this maximal response (termed EC_{5-10}) was determined. This agonist EC_{5-10} was repeatedly applied at 4 minute intervals until responses were stable. Once stable, increasing concentrations of modulator [zinc (30nM-100μM),

ethanol (10-300mM), acetone (10-1000mM) or β-hydroxybutyric acid (100uM-6mM)] were applied to the receptor in combination with the EC_{5-10} agonist, preceded by a 10 second pre-application of modulator alone. While the maximal blood alcohol limits for legally operating vehicles in many countries is 0.08% (~22mM), blood ethanol concentrations above 300 mM, while rare, have been attained and survived (O'Neill et al., 1984). Receptor modulation was assessed as $[(I_{agonist + Modulator}/I_{agonist})-1] *100$, where I is the current evoked. This experiment was repeated with 2.5mM of the zinc chelator tricine added to the bath buffer, and again at maximally effective agonist concentrations.

2.2.7 TESTING THE EFFECTS OF ACETONE AND β-HYDROXYBUTYRIC ACID IN COMBINATION WITH ETHANOL

Oocytes expressing NMDA receptors were exposed to a concentration of l-glutamine plus 10μM glycine to elicit its maximal response. The NMDAR EC_{5-10} was subsequently determined, and repeatedly applied at 4 minute intervals until stable responses were observed. Increasing concentrations of ethanol (10mM-300mM) were applied in combination with the NMDAR EC_{5-10}, and receptor modulation was calculated as described above. This experiment was repeated in the presence of Ca^{2+} free frog Ringers solution that contained either 30mM acetone or 300μM β-hydroxybutyric acid.

2.2.8 DETERMINATION OF ZINC CONTAMINATION IN ACETONE AND β-HYDROXYBUTYRIC ACID.

10mM acetone and 1mM β-hydroxybutyric acid were prepared in BD™ Falcon polypropylene tubes (Thermo Fisher Scientific, MA) containing 2% HNO_3 in ultrapure H_2O. Zinc concentrations of these two tubes plus a control tube (containing only the 2% HNO_3 in ultrapure H_2O) were determined using a quadrupole-based Agilent inductively coupled plasma mass

spectrometer (ICP-MS) at the Jackson School of Geosciences Isotope Geochemistry Facility at the University of Texas at Austin.

2.3 Results

2.3.1 THE KETONE BODIES ACETONE AND β-HYDROXYBUTYRIC ACID DECREASE NMDA-R FUNCTION

Acetone decreased NMDA receptor function in a concentration-dependent manner (**Figures 4A**, **5A** and **5B**) with threshold concentrations of approximately 10 - 30mM. Two NMDA receptor subtypes (NR1 + NR2A and NR1 + NR2B) were characterized for their sensitivities to acetone when acetone was co-applied with either a glutamate concentration producing maximal receptor activation (EC_{100}) or 5-10% maximal activation ($EC_{5\text{-}10}$). There was no significant difference between the two NMDA receptor subtypes (NR1 + 2A versus NR1 + 2B) in their sensitivities to acetone inhibition of glutamate $EC_{5\text{-}10}$ [$F(1,37)=0.51$, $p>0.48$, or EC_{100} [$F(1,26)=0.41$, $p>0.53$]. The magnitude of acetone modulation was independent of glutamate EC value (see **Figure 5** legend for statistics). β-hydroxybutyric acid was also able to negatively modulate both NMDA receptor subtypes in a concentration-dependent manner (**Figures. 4B**, **5C**, **5D**). No significant differences were found between NR1 + NR2A versus NR1 + NR2B receptor subtypes at glutamate EC_{100} [$F(1,33)=.29$, $p>0.6$]. However, a small difference was found between the two receptor subtypes using glutamate $EC_{5\text{-}10}$ [$F(1,29)=5.45$, $p<0.03$]. Receptor modulation by β-hydroxybutyric acid was dependent on glutamate concentration, with greater inhibition seen at higher glutamate concentrations (see **Figure 5** legend for statistics).

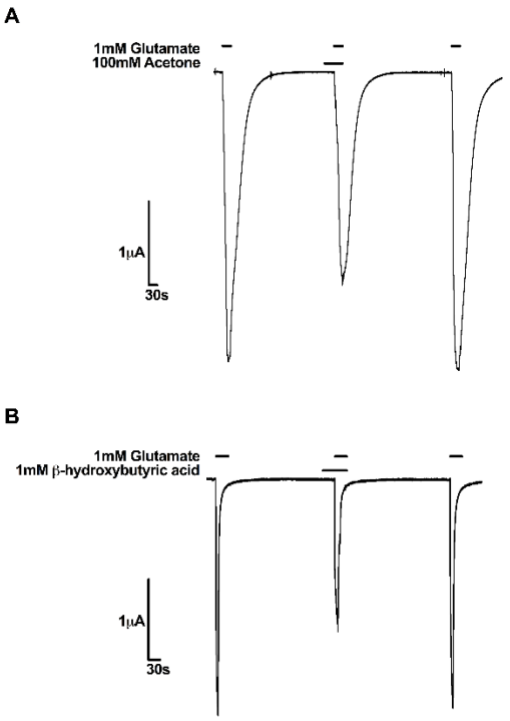

Figure 4. Sample tracings showing the effects of 100mM acetone and 100uM β-hydroxybutyric acid on NMDA NR1 + NR2A receptor function.

A. A maximally-effective concentration of 1mM glutamate (plus 10μM glycine) was applied for 30 seconds and the resulting current measured (left tracing). Pre-incubation with 100mM acetone for 10 seconds and then co-application with glutamate plus glycine for 30 sec resulted in a decreased current (right tracing). **B.** A maximally-effective concentration of 1mM glutamate with 10μM glycine was applied to the NMDA receptor without (left tracing) or with 100μM β-hydroxybutyric acid (right tracing).

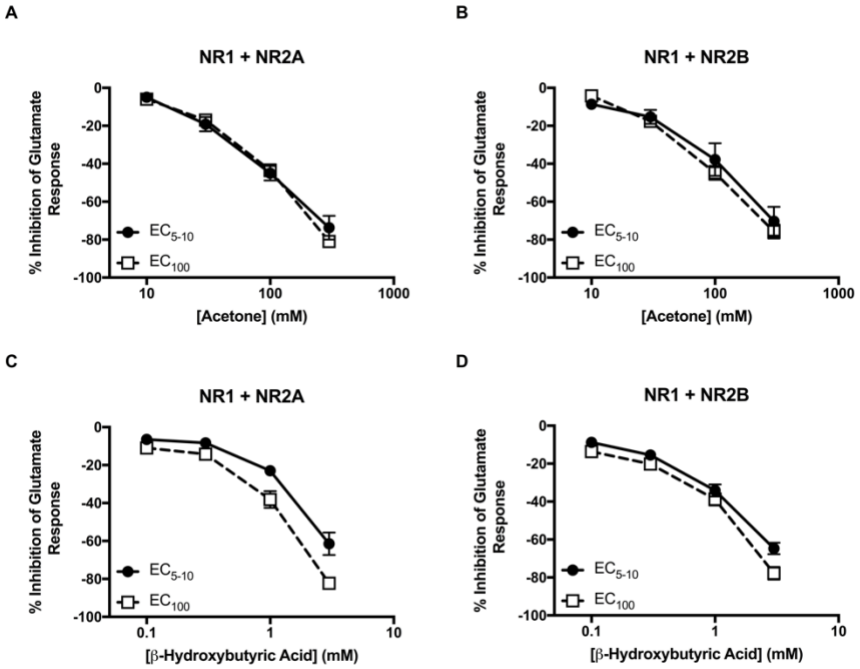

Figure 5. The ketone bodies acetone and β-hydroxybutyric acid decrease NMDA receptor function.

Acetone and β-hydroxybutyric acid effects were assayed at glutamate concentrations producing either maximal (EC_{100}, hollow squares) or 5-10% of maximal ($EC_{5\text{-}10}$, black circles) NMDA receptor responses. Each symbol represents data from 3-6 oocytes obtained from at least two separate frogs, and error bars represent the mean ± standard error. In some cases error bars fall within the symbols. **A.** The NR1 + NR2A NMDA receptor exhibited a decreased response to glutamate when exposed to increasing concentrations of acetone [$F(3, 29) = 137.9$, $p<0.0001$]. The degree of acetone inhibition was the same at $EC_{5\text{-}10}$ and EC_{100} glutamate concentrations [$F(1,29)=0.2$, $p>0.66$]. **B.** Increasing concentrations of acetone also decreased the responses of the NR1 + NR2B NMDA receptor [$F(3,34) = 59.4$, $p<0.0001$], with no effect of glutamate concentration [$F(1,34)=0.46$, $p>0.5$]. **C.** NR1 + NR2A NMDA receptor function decreased in response to increasing concentrations of β-hydroxybutyric acid [$F(3,26)= 131.5$, $p<0.0001$]. There was a significant effect of glutamate concentration, with greater β-hydroxybutyric acid effects observed when the higher glutamate concentration was used [$F(1,26) = 18.93$, $p<0.001$]. **D.** β-hydroxybutyric acid antagonized NR1 + NR2B receptor function in a concentration-dependent manner [$F(3,36)=165.7$, $p<0.0001$], and again greater inhibition was seen at higher glutamate concentrations [$F(1,36)=10.61$, $p<0.01$].

2.3.2 THE NR1 F639A MUTATION RENDERS THE NMDA RECEPTOR LESS SENSITIVE TO MODULATION BY ETHANOL, ACETONE AND β-HYDROXYBUTYRIC ACID

To confirm if the F639A amino acid substitution renders NMDA receptors less sensitive to ethanol modulation, as described in Ronald et al. (2001), ethanol (10-300mM) was applied to the NR1 + NR2A or NR1 F639A + NR2A mutant receptors in combination with a saturating glutamate concentration (**Figure 6A**). We confirmed that the F639A NR1 mutation produced a significantly reduced receptor sensitivity to ethanol (see **Figure 6** legend for statistics). To probe if acetone would also produce a weaker effect on this mutant, acetone (10 - 300mM) was applied in combination with a saturating glutamate concentration (**Figure 6B**). The F639A mutation resulted in significantly decreased receptor modulation by acetone (**Figure 6B**, hollow triangles) compared to wildtype NMDA receptors (**Figure 6B** solid circles). β-hydroxybutyric acid sensitivity was also decreased in the F639A receptors compared to wildtype receptors (**Figure 6C** hollow triangles compared to filled circles) when increasing concentrations of β-hydroxybutyric acid were applied in combination with a saturating glutamate concentration.

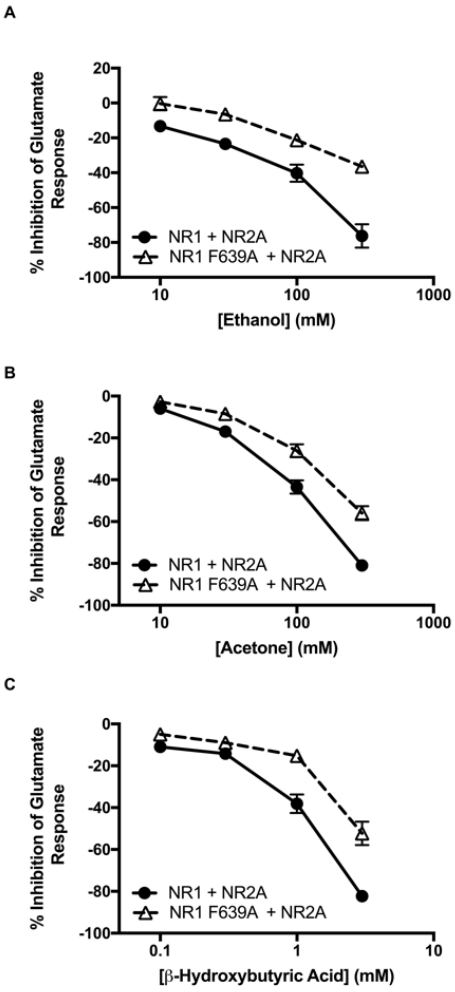

Figure 6. Effects of the NR1 F639A mutation on NMDA receptor modulation by ethanol, acetone and β-hydroxybutyric acid.

A. NR1 + NR2A NMDA receptor (black circles) function decreases when ethanol is applied in combination with a maximally-effective concentration of glutamate and glycine; in comparison the NR1 F639A + NR2A receptor mutant (hollow triangles) shows a decreased response to ethanol [$F(1,23)= 68$, $p<0.0001$] **B.** The NR1 F639A + NR2A mutant receptor (hollow triangles) also exhibits a decreased modulatory response to acetone compared to wildtype NR1 + NR2A receptors (filled circles) [$F(1,30)=32$, $p<0.0001$] **C.** The NR1 F639A + NR2A receptor (hollow triangles) also exhibits less inhibition in response to β-hydroxybutyric acid than the wildtype receptor (filled circles) [$F(1,33)=42.7$, $p<0.0001$]. Each symbol represents data from 3-6 oocytes, from at least two separate frogs, and error bars represent the mean ± standard error. In some cases error bars fall within the symbols.

2.3.3 ALCOHOL ACTS ADDITIVELY WITH KETONE BODIES AT THE NMDA RECEPTOR

Since the F639A substitution altered ethanol, acetone and β-hydroxybutyric acid sensitivities (**Figure 6**) we next tested if acetone and β-hydroxybutyric acid would compete, act additively, or act synergistically with one another. EC_{5-10} glutamate concentrations were applied to the NR1 + NR2A receptor in combination with increasing ethanol concentrations in the absence and presence of 30mM acetone (**Figure 7A**) or 300μM β-hydroxybutyric acid (**Figure 7B**). No significant differences were found in the ethanol concentration-response curves in the absence or presence of acetone or β-hydroxybutyric acid (see **Figure 7** legend for statistics). Similar results were obtained when this experiment was repeated using NR1 + NR2B receptors (data not shown). The lack of an increase or decrease in receptor modulation by ethanol in the presence of either acetone or β-hydroxybutyric acid suggests an additive role of the two compounds with ethanol. We also probed for an additive role between β-hydroxybutyric acid and acetone by applying increasing concentrations of β-hydroxybutyric acid, in combination with a saturating glutamate concentration, in the absence and presence of 30mM acetone (**Figure 7C**). The addition of acetone produced no change in the β-hydroxybutyric acid concentration-response curve, suggesting that β-hydroxybutyric acid and acetone also act additively with one another.

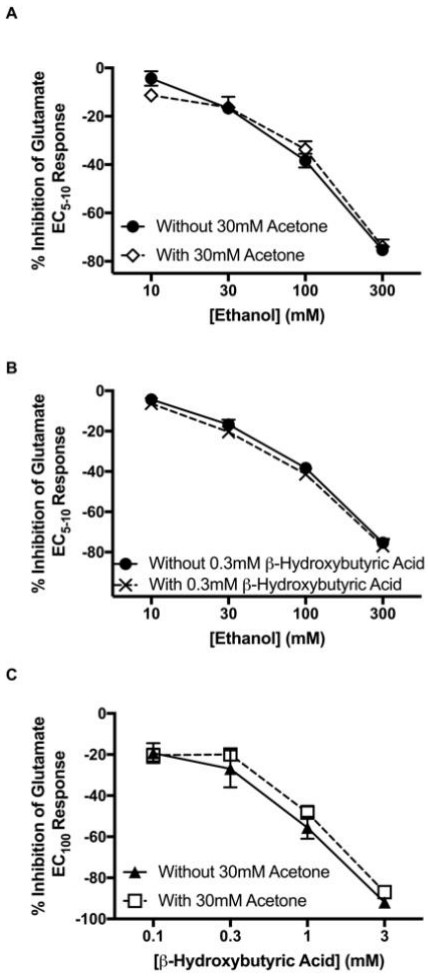

Figure 7. Effects of combining NR1 + NR2A NMDA receptor modulators with each other.

Increasing concentrations of ethanol decreased the NR1 + NR2A NMDA receptor response (filled circles) when applied to the receptor with a 5-10% maximal concentration of glutamate (EC_{5-10}) and 10μM glycine. This effect was unaltered when (**A**) 30mM acetone (hollow diamonds) [$F(3,6)=1.283$, $p>0.3$] or (**B**) 0.3mM β-hydroxybutyric acid (crosses) [$F(3,6)=0.078$, $p>0.9$] was present. In these experiments, ethanol was pre-applied with or without acetone or β-hydroxybutyric acid for 30 sec before also being co-applied with glutamate and glycine for a further 40 sec. Increasing concentrations of β-hydroxybutyric acid were pre-applied in combination with a maximal concentration of glutamate (EC_{100}) and 10μM glycine (**C**, filled triangles). This effect was unaltered in the presence of 30mM acetone (**C**, hollow squares) [$F(3,6)=0.591$, $p>0.6$]. Each symbol represents mean data from three oocytes obtained from two frogs ± the standard error. In some cases the error bars fall within the symbols.

2.3.4 ACETONE AND β-HYDROXYBUTYRIC ACID HAVE OPPOSING MODULATORY EFFECTS ON THE α1β2γ2 $GABA_A$ RECEPTOR

Concentrations of acetone greater than 100mM positively modified the effects of EC_{5-10} GABA (**Figure 8A** and **8B**, left tracings), but not saturating concentrations of GABA (**Figure 8B**, right tracings). β-hydroxybutyric acid, in contrast, acted as a negative modulator at low GABA concentrations (**Figure 8C**), with concentrations as low as 100μM producing inhibition at the receptor. This negative modulation was also dependent on the GABA concentration, as this phenomenon was not seen at saturating concentrations of GABA (**Figure 8D**).

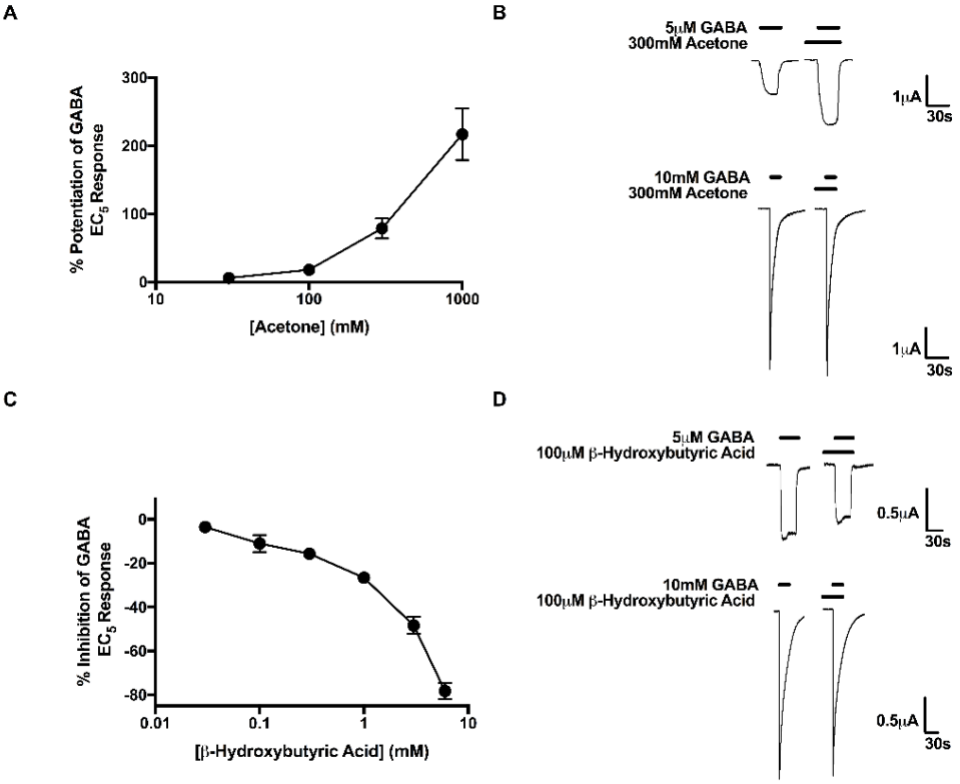

Figure 8. $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor modulation by acetone and β-hydroxybutyric acid.

A. The $GABA_A$ receptor is positively modified by acetone in a concentration-dependent manner when acetone is co-applied with a GABA concentration eliciting 5% of the maximal GABA receptor response (GABA EC_5). **B.** Acetone modulation of $GABA_A$ responses is only seen when low concentrations of GABA are used (two leftmost tracings), with no effects of acetone when it is co-applied with a maximally-effective concentration of GABA (two rightmost tracings). **C**. β-hydroxybutyric acid acts as a negative modulator of the $GABA_A$ receptor, but is only able to exert its actions when applied in combination with low concentrations of GABA (**D**, two leftmost tracings) and not when applied in combination with a saturating concentration of GABA (**D**, two rightmost tracings). Each symbol represents data collected from 4-8 oocytes, with error bars representing the mean ± standard error. The error bars fall within some symbols.

2.3.5 ACETONE IS A WEAK POSITIVE ALLOSTERIC MODULATOR AT THE α1 GLYCINE RECEPTOR, AND THIS MODULATION IS DEPENDENT ON THE SIZE OF THE AMINO ACID RESIDUE OCCUPYING POSITION 267

Acetone affected the wildtype glycine receptor only at high concentrations (>300mM) (**Figure 9A**). The magnitude of this modulation was unchanged in the presence of the zinc chelator tricine (see **Figure 9** legend for statistics), indicating that glycine receptor modulation by acetone is not zinc dependent, unlike other modulators of the glycine receptor such as ethanol. To confirm that acetone modulation of the receptor is not zinc dependent, acetone was applied in combination with glycine to the W170S α1 receptor (**Figure 9A**). No significant difference was found in W170S receptor versus wildtype receptor modulation by 1M acetone (see **Figure 9** legend for statistics). To test if mutating residue S267 of the glycine receptor would similarly affect ethanol and acetone potentiation, 200mM ethanol or 1M acetone was added in combination with glycine $EC_{5\text{-}10}$ to wildtype α1 or S267A, S267I or S267Y α1 glycine receptors. Increasing the size of the residue occupying position S267 decreased the magnitude of receptor potentiation by ethanol, and also decreased receptor potentiation by 1M acetone (**Figure 9B**). In the S267Y α1 glycine receptor both ethanol and acetone inhibited receptor function.

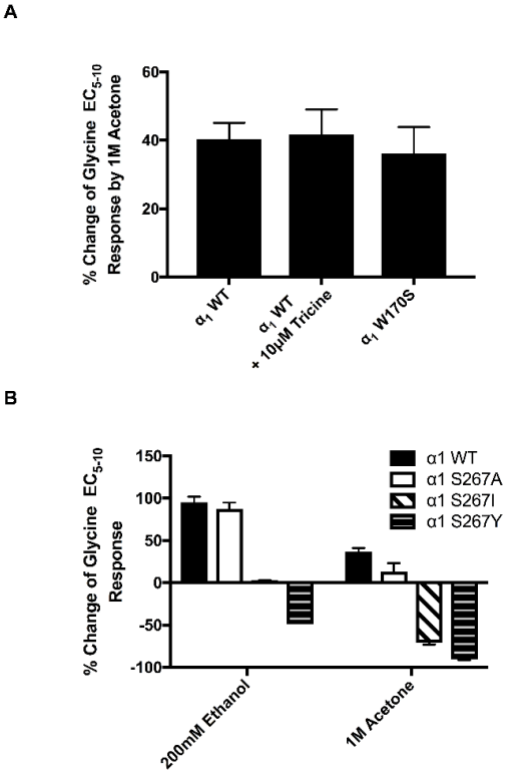

Figure 9. Acetone acts as a weak positive allosteric modulator at the α_1 glycine receptor.

A. 1M acetone enhanced α1 glycine receptor function when applied to the receptor in combination with a glycine concentration capable of 5-10% receptor activation (glycine $EC_{5\text{-}10}$). Acetone was pre-applied to the oocyte alone for 10 seconds before also being applied with glycine for 30 sec. The addition of the zinc chelator tricine to the buffer did not change glycine receptor modulation by glycine. Application of acetone in combination with glycine to the W170S α1 glycine receptor mutant, a receptor which does not respond to the enhancing effects of zinc, also produced no change in receptor modulation by acetone. A one-way ANOVA revealed no significant differences among the three conditions [$F(2,15)=0.18$, $p>0.83$]. Each bar represents data from 6 oocytes from at least 2 frogs, with error bars representing the standard error of the mean. **B.** The amino acid size at position S267 of the glycine receptor affects ethanol potentiation, with increasing size resulting in decreased potentiation by ethanol. A one-way ANOVA revealed a significant effect of receptor mutation on ethanol modulation [$F(3,13)=65.03$, $p<0.0001$], and a Dunnett's multiple comparisons test found that the S267I and S267Y receptor mutant responses were significantly different from those of wildtype glycine receptors. Altering amino acid size at position 267 also significantly affected glycine receptor modulation by 1M acetone [$F(3,30)=98.73$, $p<0.0001$], with Dunnett's multiple comparisons tests showing significant differences in acetone modulation by all S267 mutants compared to wildtype receptors. Each bar represents data obtained from 3-8 oocytes from at least 2 frogs, ± the standard error of the mean.

2.3.6 β-HYDROXYBUTYRIC ACID IS A NEGATIVE MODULATOR OF THE GLYCINE RECEPTOR

β-hydroxybutyric acid inhibited glycine receptor function, in a concentration-dependent fashion (**Figure 10A)** and slightly greater inhibition was seen in the presence of the zinc chelator tricine (**Figure 10A**, hollow circles). Zinc acts as a biphasic modulator at glycine receptors, increasing glycine receptor function at nanomolar concentrations, and decreasing receptor function at higher, micromolar, concentrations (**Figure 10B**, filled circles). The W170S glycine receptor is insensitive to the potentiating effects of zinc, but retains the negative modulation produced by higher concentrations of zinc (**Figure 10B**, filled triangles). In contrast, the H109A glycine receptor is less sensitive than wildtype receptors to the inhibitory effects of zinc (**Figure 10B**, filled squares). The wildtype, W170S and H109A mutant receptors all significantly differed from each other in their responses to zinc (see **Figure 10** legend for statistics). The addition of tricine to W170S receptors did not produce a significant change in W170S receptor modulation by β-hydroxybutyric acid (see **Figure 10** legend for statistics). The H109A mutant receptor converted β-hydroxybutyric acid from a negative to a positive allosteric modulator and this showed some dependence on zinc (**Figure 10D**).

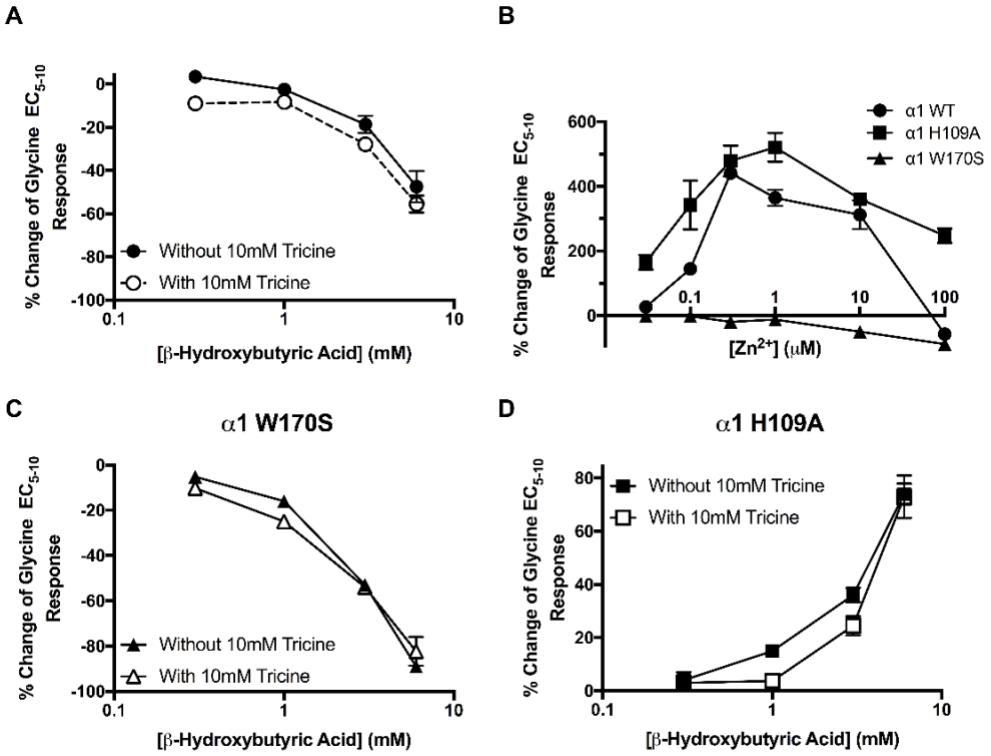

Figure 10. β-hydroxybutyric acid is a negative modulator of the α_1 glycine receptor.

A. β-hydroxybutyric acid inhibits the effects of the glycine $EC_{5\text{-}10}$ in a concentration-dependent manner (solid circles). Chelation of zinc using 10mM tricine had no overall significant effect on receptor potentiation by β-hydroxybutyric acid (hollow circles) [$F(1,49)=15.57$, $p<0.001$] **B.** Mutations in the α1 glycine receptor variably affect its responses to increasing zinc concentrations. The α1 wildtype receptor exhibits a biphasic response to zinc (solid circles), exhibiting enhancement at low μM zinc concentrations and inhibition as the zinc concentration is increased. The H109A mutation is less sensitive to the inhibitory effects of zinc (solid squares), while the W170S mutation renders the receptor insensitive to the potentiating effects of zinc (solid triangles). **C.** The W170S receptor retained sensitivity to the inhibiting effects of β-hydroxybutyric acid, the effect of which was unaffected by the addition of the zinc chelator tricine (hollow triangles) [$F(1,11)=1.76$, $p>0.21$]. **D**. β-hydroxybutyric acid is a positive modulator at the H109A α1 glycine receptor. Increasing concentrations of β-hydroxybutyric acid were co-applied with a glycine $EC_{5\text{-}10}$ in the absence (solid squares) and presence (hollow squares) of 10mM tricine. There is a significant difference in the effect of β-hydroxybutyric acid in the presence of tricine (ANOVA [$F(1,8)=31.49$, $p<0.001$]). Each symbol represents data obtained from 3-8 oocytes from at least 2 frogs, ± the standard error of the mean.

2.3.7 INVESTIGATING ZINC CONTAMINATION IN ACETONE AND β-HYDROXYBUTYRIC ACID STOCKS USING ICP-MS

ICP-MS measurements revealed that a control polypropylene tube containing 2% HNO_3 and ultrapure water had zinc levels (± standard deviation) of 3.275±0.086 ppb, 1mM β-hydroxybutyric acid contained 3.455±0.019 ppb, and 10mM acetone contained 3.258±0.071. This correlates to approximately 50nM zinc present in distilled water in polypropylene tubes, with no further contamination in acetone. However, the tube containing 1mM β-hydroxybutyric acid contained approximately 3nM of additional zinc. These zinc concentrations could be high enough to explain, at least in part, the decreases in β-hydroxybutyric acid effects seen in **Figures 10A** and **10D** in the presence of tricine.

2.4 Discussion

The present study demonstrates that acetone and β-hydroxybutyric acid affect NMDA, $GABA_A$ and glycine receptor function. β-hydroxybutyric acid modulates NMDA, $GABA_A$ and glycine receptors at physiologically-relevant concentrations. Typically, ketone body concentrations remain below 100 μM but starvation can lead to plasma concentrations of 1mM acetone, while blood β-hydroxybutyrate levels can exceed 5mM (Crandall, 1941; Mitchell et al., 1998; Owen, 2006). Brain concentrations of β-hydroxybutyrate after just three days of fasting in humans have been reported to be approximately 1mM, and cerebrospinal fluid levels in children following a ketogenic diet are approximately 400μM (Nordli and De Vivo, 1997; Pan et al., 2000). In patients suffering from diabetic ketoacidosis β-hydroxybutyrate and acetone levels can total more than 25mM (Malchoff et al., 1984), most of which will be β-hydroxybutyric acid.

Concentrations of acetone greater than 10mM affected both NR1+NR2A and NR1+NR2B NMDA receptors (**Figures 5A**, **5B**). This modulation was independent of glutamate concentration, as acetone produced similar effects when applied with both high and low concentrations of glutamate. This suggests that acetone acts as a non-competitive allosteric modulator at the NMDA receptor in a manner similar to alcohol (Peoples et al., 1997). β-hydroxybutyric acid modulated NR2A- and NR2B-containing NMDA receptors to a similar extent, but unlike acetone the receptor modulation by β-hydroxybutyric acid appeared to be somewhat dependent on glutamate concentration, with greater effects of β-hydroxybutyric acid seen when it was applied with a high concentration of glutamate (**Figures 5C** and **5D**). This action is consistent with that of an uncompetitive antagonist, as also exemplified by memantine (Chen and Lipton 1997). Uncompetitive antagonists require prior receptor activation by a receptor agonist (in this case glutamate) before they can act. In contrast to our results, Yang et al. (2007) reported no β-

hydroxybutyric acid modulation of NR1+NR2A NMDA receptors expressed in *Xenopus* oocytes, but they did not state what β-hydroxybutyric acid concentrations they tested.

The substitution of phenylalanine 639 with alanine in the NR1 subunit rendered the NMDA receptor less sensitive to ethanol (**Figure 6A**), consistent with previous findings (Ronald et al., 2001). This mutation also decreased the sensitivity of the NMDA receptor to both acetone (**Figure 6B**) and β-hydroxybutyric acid (**Figure 6C**), suggesting that this mutation is interfering with the actions of all three modulators. It has been proposed that the F639 residue is important for the coupling of agonist binding to receptor opening, and other modulators of the NMDA receptor besides ethanol, such as isofluorane, xenon and chloroform also produce less inhibition in these mutant receptors, compared to wildtype (Ogata et al., 2006, Yang et al., 2007). To investigate the possibility that acetone and/or β-hydroxybutyric acid were competing with alcohol at the same site (i.e. some shared site involving the F639 residue), ethanol concentration-response curves were generated in the absence and presence of either acetone or β-hydroxybutyric acid (**Figure 7**). Neither 30mM acetone nor 0.3mM β-hydroxybutyric acid produced changes in the ethanol concentration-response curves in NR1 + NR2A or NR1 + NR2B receptors, suggesting that there was no competition for ethanol at these fixed concentrations of ketone bodies. If there was competition or synergy between ethanol and either of the ketone bodies we would hypothesize that at least one of the ethanol concentrations tested would be significantly different in the presence of a ketone. The similar sensitivities to ethanol in the absence and presence of ketone bodies suggests that these compounds act additively with one another. In addition, 30mM acetone did not significantly affect the degree of 0.1-3mM β-hydroxybutyric acid inhibition of NMDA receptor function (**Figure 7C**).

Compounds that act additively with ethanol may be of therapeutic interest for the targeting of alcohol dependence and/or withdrawal. These compounds would be expected to make individuals more susceptible to the effects of alcohol at lower concentrations, thus potentially decreasing the number of drinks they consume. Additionally, since acetone and ketone produce similar degrees of inhibition of the NMDA receptor as alcohol, switching alcoholics to a ketogenic diet while they withdraw from alcohol may help alleviate withdrawal symptoms. Rodents fed a ketogenic diet while consuming alcohol for six days exhibited milder alcohol withdrawal symptoms compared to rodents fed a regular diet (Dencker et al., 2018). It would be of interest to determine the magnitude of withdrawal symptoms in humans and rodents administered a ketogenic diet only after alcohol withdrawal, not before as well as after as modeled by Dencker et al. (2018), as a type of substitution therapy. The National Institute on Alcohol Abuse and Alcoholism is currently conducting such a three-year clinical trial (Clinical trials gov identifier NCT03255031) in 100 participants assessing the benefits of the ketogenic diet following alcohol withdrawal.

Acetone also acts in a manner similar to alcohol on $GABA_A$ and glycine receptors. High, non-physiological (>100mM) concentrations of acetone positively modified $GABA_A$ and glycine receptors, with $GABA_A$ receptors showing greater effects of 1M acetone than glycine receptors (**Figures 8A** and **9A**). Our findings contrast with those of Yang et al. (2007) who reported that acetone was a potent positive modulator of homomeric $\alpha 1$ glycine receptors (at concentrations higher than 50μM), and to a lesser degree also of $\alpha 1\beta 2\gamma 2s$ $GABA_A$ receptors (at concentrations exceeding 50mM). The Yang et al., (2007) study found that acetone modulation of the glycine receptor peaked at 2.5mM acetone, with higher concentrations higher resulting in smaller effects; acetone exhibited an inverted U-shaped concentration response curve. The acetone concentration-response curve shown in Yang et al. (2007) is reminiscent of the zinc concentration-response curve seen in glycine receptors, where low zinc concentrations positively modulate the receptor by

binding to a zinc potentiation site, while higher zinc concentrations occupy a lower affinity zinc inhibitory site, resulting in decreased receptor potentiation and ultimately receptor inhibition (as shown in **Figure 10B**, filled circles). One possibility is that in the Yang et al. (2007) study there may have been sufficient zinc contamination in the acetone stock or glassware to produce the data observed. A previous study in our lab showed that zinc contamination may be a common occurrence (Cornelison and Mihic, 2014).

To test if the modulatory effect of acetone on glycine receptors that we observed might be the result of contaminating zinc, or if acetone acted on the glycine receptor in a zinc-dependent manner, we tested the effects of 1M acetone on wildtype glycine receptors in the presence of a zinc chelator, tricine, and on mutant W170S α1 glycine receptors in which amino acid tryptophan 170 was changed to serine (**Figure 9A**). The presence of tricine did not alter acetone enhancement of the glycine receptor, suggesting that there was little to no zinc contamination present in our acetone stock, and that acetone actions on the glycine receptor are not zinc dependent, similar to volatile anesthetics (McCracken et al., 2010). This contrasts with other modulators of the glycine receptor, such as alcohol and inhalants such as trichloroethane, which are influenced by zinc (McCracken et al., 2010; Cornelison et al., 2017). The W170S glycine receptor mutant is insensitive to the potentiating effects of zinc (**Figure 10B,** filled triangles), as previous studies have described (Cornelison et al., 2017; Zhou et al., 2013). If the acetone stock solution used in our studies had zinc contamination, or acted in a zinc-dependent manner, one would expect to see decreased effects of acetone on the W170S receptor. However, acetone produced a similar modulatory effect in W170S receptors as in wildtype receptors (**Figure 9A**), suggesting that the acetone effects observed on wildtype glycine receptors are not the result of contaminating zinc, nor are they zinc dependent. ICP-MS results confirmed that there was no zinc contamination in our acetone stock. Interestingly, the effects of acetone appeared to be dependent on the amino acid

present at position 267 of the glycine receptor (**Figure 9B**). This is similar to how amino acid size at position 267 affects ethanol potentiation and is unsurprising given the similarity in structure between acetone and alcohol; the replacement of acetone's carbonyl group with a carboxyl group would yield isopropyl alcohol (Ye et al., 1998).

β-hydroxybutyric acid concentrations greater than 100μM inhibited the effects of GABA on $GABA_A$ receptors (**Figure 8C**), while concentrations over 1mM negatively affected glycine receptor function (**Figure 10A**). This is also in contrast to the Yang et al. (2007) study, which found that β-hydroxybutyric acid positively modified glycine receptors at β-hydroxybutyric acid concentrations greater than 3mM and produced around 14% enhancement of $GABA_A$ receptors at a β-hydroxybutyric acid concentration of 20mM. The effects of β-hydroxybutyric acid on glycine receptors seen by Yang et al. (2007) might also be attributed to zinc contamination, but we are unable to speculate why they saw no inhibition with β-hydroxybutyric acid, as high levels of contaminating zinc would inhibit, not potentiate, $GABA_A$ receptors. While our ICP-MS results suggest a small amount of zinc contamination in our β-hydroxybutyric acid stock solution, zinc chelation by tricine resulted in a greater inhibitory effect of the glycine receptor current by β-hydroxybutyric acid (**Figure 10A**). This result was not unexpected, as low levels of contaminating zinc would tend to potentiate at the glycine receptor.

The positive modulation by acetone, and negative modulation by β-hydroxybutyric acid on glycine and $GABA_A$ receptors appears to be dependent on agonist (GABA or glycine) concentration, with acetone and β-hydroxybutyric acid having modulatory effects only at low concentrations of agonist (**Figure 8B**, **8D**, glycine data not shown). This is similar to other modulators of these receptors, such as benzodiazepines on $GABA_A$ receptors and zinc or ethanol on glycine receptors (Newland et al.,1991; Farley and Mihic, 2015).

To our knowledge, this is the first report that β-hydroxybutyric acid negatively modulates NMDA, $GABA_A$ and glycine receptors at pharmacologically-relevant concentrations. If β-hydroxybutyric acid inhibits both excitatory and inhibitory neurotransmission, one might surmise that the two effects could cancel each other out *in vivo*, thus questioning the clinical relevance of our findings. However, we found greater percent effects of β-hydroxybutyric acid on NMDA than $GABA_A$ and glycine receptors (compare **Figures 5**, **8** and **10**). Thus, although antagonism of agonist effects on inhibitory neurotransmitter receptors would to some extent counteract the β-hydroxybutyric acid inhibition observed at NMDA receptors, this agent appears more efficacious at the latter. It should also be pointed out that even if percent inhibition by β-hydroxybutyric acid was the same in $GABA_A$ and NMDA receptors, this does not mean that the two effects would necessarily cancel each other out. Also playing roles would be the relative numbers of each receptor type on individual neurons, how quickly the receptors desensitized, how often they were activated relative to one another, and what their relative unitary conductances were, among other factors. None of the receptors tested responded to physiological concentrations (1mM) of acetone, but NMDA receptors had the lowest threshold (10mM) for acetone effects of the receptors tested. The lack of clinical data describing acetone concentrations achieved in the brain during ketogenesis may account for a lower physiological acetone concentration reported than what truly occurs. We hypothesize that ketogenesis can yield β-hydroxybutyric acid, but not acetone, concentrations *in vivo* sufficient to modulate NMDA, $GABA_A$ and glycine receptor function, and that this receptor modulation may account in part for the therapeutic effects seen with a ketogenic diet.

CHAPTER 3: AN INTER-SUBUNIT ELECTROSTATIC INTERACTION IN THE $GABA_A$ RECEPTOR FACILITATES ITS RESPONSES TO BENZODIAZEPINES[2]

3.1 Introduction

Inter- and intra-subunit electrostatic interactions play important roles in cys-loop receptor function. For example, electrostatic interactions between residues of adjacent alpha subunits in the glycine receptor play an important role in its activation (Todorovic et al., 2010). Specifically, the aspartate 97 residue is thought to interact with arginine 119 to stabilize the closed state of the glycine receptor, and once this bond is broken after agonist binding, the channel opens. Additionally, electrostatic interactions between aspartic acid 149 and lysine 279 within the same α subunit, as well as between aspartic acid 146 and lysine 215 within the same β subunit are implicated in the coupling of GABA binding to the opening of the $GABA_A$ receptor (Kash et al., 2003; Kash et al., 2004). Further, glutamic acid 153 and lysine 196 within the same β subunit of the $GABA_A$ receptor may be involved in stabilizing the open state of the receptor (Venkatachalan and Czajkowski, 2008). Disulfide trapping experiments have led to insights into the conformational changes that benzodiazepines produce in the $GABA_A$ receptor after binding; however, thus far an electrostatic interaction has not been identified in the $GABA_A$ receptor that occurs because of this conformational change (Hanson and Czajkowski, 2011).

[2] This chapter details work that has been published. Pflanz N.C. was responsible for the design, conduction and analysis of these experiments and for writing the manuscript.
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In the current chapter, we used homology modeling with published structures to produce models of $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor. We used these models to identify potential electrostatic interactions occurring before or after the conformational changes produced by benzodiazepine binding, identifying a pair of residues that appear to be interacting in a manner specific for benzodiazepine modulation of the $GABA_A$ receptor.

3.2 Methods

3.2.1 Reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated below.

3.2.2 Structural modeling

Two homology models of the $GABA_A$ receptor were generated using the Modeler module of Discovery Studio 2016 (DS 2016, Biovia, San Diego, CA) as previously described (McCracken et al., 2016). The first model was of the $GABA_A$ receptor in the benzodiazepine-unbound state. This was built using the GluCl X-ray structure in the absence of ivermectin as a template (Yoluk et al., 2013). This template was produced by starting with the structure of $GABA_A$ receptor with five ivermectin molecules bound (PDB ID 3RHW), removing the five ivermectin molecules, and then running extensive constrained molecular dynamics simulations using GROMACS 4.5. The resulting model was judged to be in the closed/resting state because the subunits moved closer by 2.0 Å and the pore diameter decreased by 1.2 Å (Yoluk et al., 2013). The second homology model illustrated the $GABA_A$ receptor after diazepam was bound. This model was based on a GluCl/ELIC X-ray structure which modeled diazepam binding (Bergmann et al., 2013). It should

be noted that other investigators have proposed a different orientation of diazepam docking at this α/γ interface (Ci et al., 2008). The latter model is based primarily on the glutamate-bound GluCl crystal structure (PDB ID 3RIF) with a contribution of the ELIC crystal structure (PDB ID 2VLO). Of interest for the present results; in $GABA_A$ receptor α_1, lysine 104 is in beta strand 4 and all coordinates are from GluCl. However, in $GABA_A$ receptor γ_2, aspartic acid 75 is in beta strand 2, a residue that is conserved in ELIC but not in GluCl, As a result, the investigators used the ELIC structure as a template for residues 75-77 (Bergmann et al., 2013).

Since both templates are homopentamers and our goal was to measure intersubunit interactions, we prepared a composite sequence by linking $GABA_A$ receptor $\alpha_1/\beta_2/\alpha_1/\beta_2/\gamma_2$ and aligning the composite with the sequence of the two templates (Trudell, 2002). Then the $GABA_A$ receptor sequences were trimmed to match the length of the template sequences as needed. The two pairs of aligned sequences were submitted to the Modeler module of DS 2016.

Both of the resulting homology models were assigned the CHARMM force field in DS 2016, minimized, and then subjected to molecular dynamics simulations at 300K as previously described (McCracken et al., 2016). These two models were analyzed for possible electrostatic interactions using DS 2016.

3.2.3 SITE-DIRECTED MUTAGENESIS

Human cDNAs encoding α_1, β_2 and γ_2 $GABA_A$ receptor subunits, subcloned into a pBK-CMV vector, were used in this study. Point mutations were introduced in the α_1 and γ_2 subunits using a QuikChange site-directed mutagenesis kit (Agilent Technologies, CA). These mutations were confirmed with double-stranded DNA sequencing.

3.2.4 TWO-ELECTRODE VOLTAGE CLAMP ELECTROPHYSIOLOGY

Oocytes were harvested and injected as described in Chapter two. Oocytes expressed $GABA_A$ receptors 1-3 days post injection with cDNA, and all electrophysiological recordings were completed within this time. An oocyte was placed in a 100µL bath containing ND-96 buffer and continuously perfused at a rate of 2ml/minute, as described in chapter two.

3.2.5 CONCENTRATION-RESPONSE CURVE GENERATION AND ANALYSIS

Concentration-response data were collected for wildtype $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor or the α_1(K104C)$\beta_2\gamma_2$, α_1(K104A)$\beta_2\gamma_2$, $\alpha_1\beta_2\gamma_2$(D75C), $\alpha_1\beta_2\gamma_2$(D75A), α_1(K104C)$\beta_2\gamma_2$(D75C), α_1(K104A)$\beta_2\gamma_2$(D75A) or α_1(K104D)$\beta_2\gamma_2$(D75K) mutants. Once voltage clamped, the oocyte was exposed to a maximally-effective concentration of GABA (100mM) for 10 sec. Following a ten-min washout with ND-96 buffer to allow re-sensitization of the receptors, increasing concentrations of GABA (3µM-10mM) were applied for 20-30 seconds, allowing 5-10 minutes of washout between applications. Another maximally-effective concentration of GABA (100mM) was applied at the end of the experiment, so that any drift (up or down) of current throughout the experiment could be corrected for. The responses to increasing concentrations of GABA were fit to the Hill equation using SigmaPlot 11.0 (Systat Software, CA).

3.2.6 $GABA_A$ RECEPTOR MODULATOR RESPONSES

Responses to modulators (1µM diazepam, 1µM flunitrazepam, 1µM flumazenil, 1µM Ro 15-4513, 1µM zolpidem, 100nM and 1µM allopregnanolone, 200mM ethanol) were recorded in oocytes expressing wildtype or mutant receptors. 10mM stock solutions of all modulators (made

with 0.1% DMSO in ND-96 buffer), except ethanol, were stored at -20C and diluted in ND-96 before use.

The GABA EC_{5-10}, the concentration of GABA that produces 5-10% of the maximal response, was first determined and then repeatedly applied for 30 seconds, followed by 3 minute ND-96 washouts, until responses were stable. Once stable, oocytes were pre-incubated for 30 seconds with a modulator, followed immediately by a co-application of modulator plus GABA EC_{5-10}. The allosteric modulation was calculated as $[(I_{GABA + Modulator}/I_{GABA})-1] *100$.

3.2.7 DITHIOTHREITOL (DTT) AND HYDROGEN PEROXIDE (H_2O_2) TREATMENT

DTT and H_2O_2 were made fresh in ND-96 buffer before each experiment. The GABA EC_{5-10} was determined and applied at three minute intervals until stable responses were obtained. This was repeated after a 2 minute DTT (2mM) application, during which the oocyte was unclamped from -80 mV, and given a subsequent 5 minute ND96 washout, and after a 90 second application of 0.3% H_2O_2 (7 minute washout after, oocyte also unclamped). To measure the effects of DTT and H_2O_2 on allosteric modulation, the GABA EC_{5-10} was determined and ensured to be stable. GABA was then applied in the presence of an allosteric modulator, as described previously.

3.2.8 PROPYL METHANETHIOSULFONATE (PMTS) TREATMENT

A 300mM PMTS (Toronto Research Chemicals, Canada) stock solution in DMSO was stored at -20°C and diluted to 0.5mM in ND-96 before each experiment. The GABA EC_{5-10} was determined and applied at 3 minute intervals until stable responses were observed. Oocytes were then unclamped from -80mV and treated with 0.5mM PMTS for 60 seconds. After a 2 minute

wash, oocytes were re-clamped to -80 mV and the same GABA EC_{5-10} was reapplied. Percent changes in current were calculated as $[(I_{GABA\ after\ PMTS}/I_{GABA\ before\ PMTS})-1] *100$. This was repeated after a 2 minute treatment with 2mM DTT, waiting 5 or 60 minutes after application before applying PMTS.

3.3 Results

3.3.1 Molecular modeling identifies possible electrostatic interactions present before and after benzodiazepine binding at the α_1-γ_2 subunit interface of the $GABA_A$ receptor.

As a starting point for our studies, we used two different models to identify potential electrostatic interactions at the α_1-γ_2 subunit interface. The first was based on molecular dynamic modeling performed by Yoluk et al. (2013) on the GluCl ligand-gated cys-loop receptor, in the absence of ivermectin (**Figure 11, left**). This first model corresponds to the closed, GABA- and benzodiazepine-unbound state of the $GABA_A$ receptor in our studies. The second model (**Figure 11, right**) is based on the GABA- and diazepam-bound $GABA_A$ receptor model described by Bergmann et al. (2013). Choosing to investigate only charged residues predicted to be 6Å or less apart, we identified seven interactions that could occur before benzodiazepine binding, as well as four interactions that could occur after benzodiazepine binding. Two of these pairs, aspartic acid 56 of the α_1 subunit (α_1D56) with arginine (R) 197 of the γ_2 subunit (γ_2R197), as well as glutamic acid (E) 58 of the α_1 subunit (α_1E58) with γ_2R197, were predicted to form electrostatic pairs both before and after diazepam binding. In the present study, we focused on the electrostatic interactions that were predicted to interact closest to the benzodiazepine binding site (**Figure 11, interactions B-F** and **H-K**).

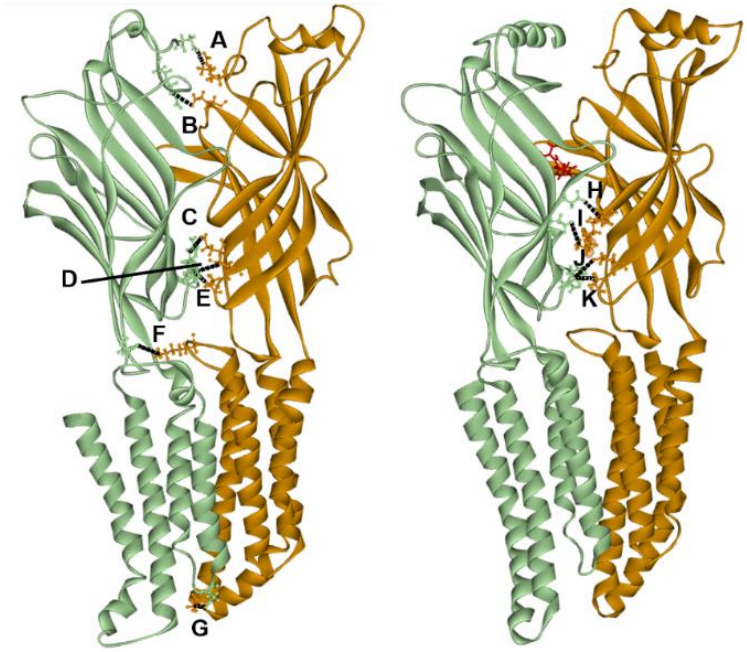

Figure 11. Two different homology models of the α_1 (orange) - γ_2 (green) interface of the $GABA_A$ receptor.

The model on the left is based on a modified ivermectin-unbound GluCl crystal structure and represents the GABA-unbound closed state of the channel (Yoluk et al., 2013). The model on the right is based on the glutamate-bound GluCl crystal structure with a contribution of the ELIC crystal structure and represents the diazepam (in red) bound receptor (Bergmann et al., 2013). Both models depict the inside of the interface. Labeled interactions represent putative electrostatic interactions of residues 6Å or less apart that are predicted to occur between residues in the α_1 and γ_2 subunits before (A-F) or after (H-K) diazepam binding. **A**: α_1R28-γ_2D26; 5Å. **B**: α_1E165-γ_2R97; 4Å. **C**: α_1E137-γ_2R194; 5Å. **D**: α_1E58-γ_2R197; 5Å. **E**: α_1D56-γ_2R197; 5Å. **F**: α_1K278-γ_2D161; 5Å. **G**: α_1K311-γ_2D260; 3Å. **H**: α_1K105-γ_2D120; 5Å. **I**: α_1K104-γ_2D75; 5Å. **J**: α_1E58-γ_2R197; 5Å. **K**: α_1D56-γ_2R197; 6Å. Black dashed lines represent inter-subunit bonds.

3.3.2 EFFECTS OF CYSTEINE SUBSTITUTION ON DIAZEPAM POTENTIATION OF $GABA_A$ RECEPTOR FUNCTION

Diazepam (1μM) enhancement of the effects of a GABA concentration required to produce 5-10% of the maximal response (EC_{5-10}), was tested on a series of cysteine mutants. Cysteine substitution of residues of the α_1 or γ_2 subunit predicted to be involved in electrostatic interactions before and/or after diazepam binding resulted in a significant effect of mutation on diazepam potentiation (see **Figure 12** legend for statistics). Replacing α_1 E58 with cysteine [α_1(E58C)] resulted in a significant increase in diazepam potentiation, while the α_1(K104C), α_1(E137C), γ_2(D75C) and γ_2(R197C) substitutions all resulted in significant decreases in diazepam enhancement (**Figure 12**). Six of the other residues substituted with cysteine, α_1(D56C), α_1(K105C), α_1(E165C), γ_2(R97C), γ_2(D120C) and γ_2(R194C), resulted in no significant changes in receptor enhancement by diazepam, compared to wildtype $GABA_A$ receptor. Of the pairs probed, the only hypothesized pair that produced similar changes in diazepam effects upon mutation to cysteine were α_1(K104C) and γ_2(D75C) (**Figure 11, interaction I**). If an electrostatic interaction was occurring between two residues, one would expect similar changes in receptor function if that bond was broken by mutating either residue. For this reason we focused on the α_1K104 - γ_2D75 pair. Before diazepam binding α_1K104 was predicted to be approximately 9Å from γ_2D75 (**Figures 13A** and **13B**), but after diazepam binding these residues were predicted to move much closer together, to approximately 5Å apart (**Figure 13C**).

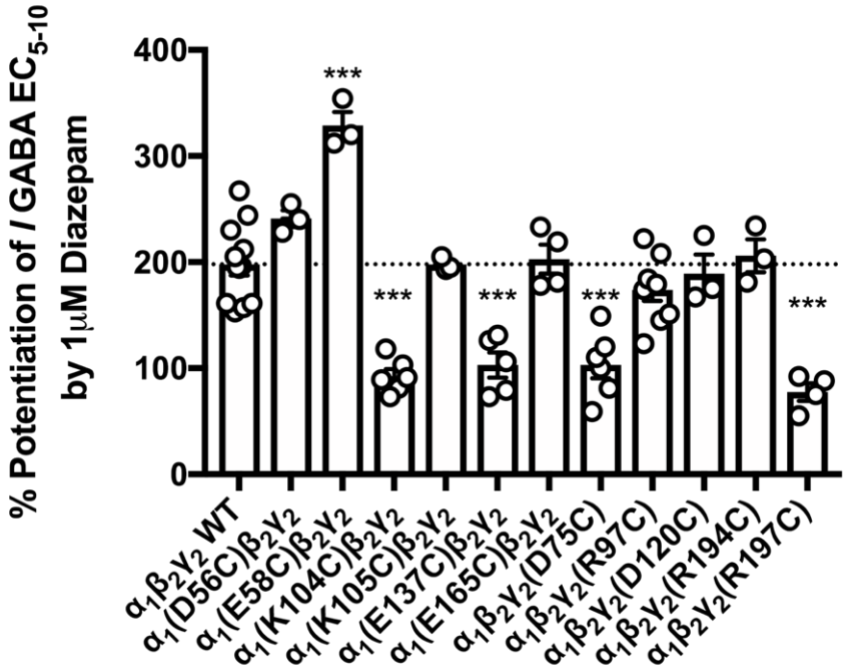

Figure 12. Diazepam enhancement of $GABA_A$ receptor function is altered in some cysteine mutations of residues predicted to form electrostatic interactions at the α_1-γ_2 subunit interface.

$EC_{5\text{-}10}$ GABA was applied alone, as well as in the presence of 1μM diazepam, to wildtype and multiple cysteine substituted receptors. The horizontal dashed line indicates the level of potentiation produced by diazepam in wildtype receptors. A one-way ANOVA showed a significant effect of cysteine substitution on receptor enhancement by 1μM diazepam, [$F(11,60)=26.310$, $p<0.001$]. A post-hoc Tukey's test showed a significant change ($p<0.001$) in potentiation by 1μM diazepam in α_1E58C, α_1K104C, α_1E137C, γ_2D75C and γ_2R197C containing $GABA_A$ receptor. Each symbol represents the percent potentiation of the GABA $EC_{5\text{-}10}$ by one oocyte, and each bar represents the mean percent potentiation ± SEM.

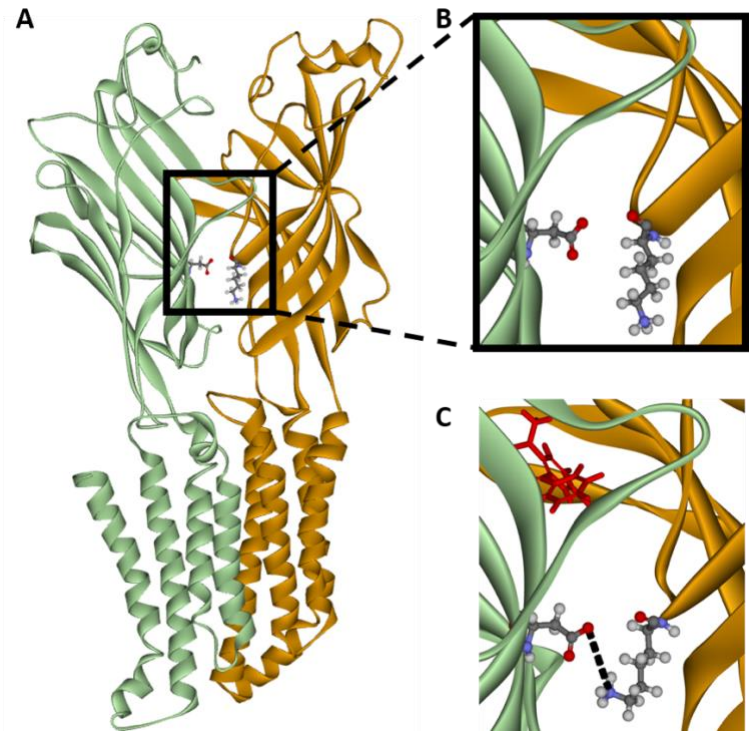

Figure 13. Homology models of the α_1 (orange) - γ_2 (green) interface inside the $GABA_A$ receptor in both the GABA-unbound closed state of the channel and the diazepam-bound open state of the channel.

These models predict that the nitrogen atom of α_1 lysine 104 (orange residue) and oxygen atom of γ_2 aspartic acid 75 (green residue) are within 9Å of each other before GABA and diazepam bind (**A** and enlarged in **B**) but move to within 5Å of each other after GABA and diazepam (in red) bind to the receptor (**C**).

3.3.3 Effects of cysteine substitution on GABA sensitivity at α_1K104 and γ_2D75 residues

GABA concentration-response curves for α_1(K104C)$\beta_2\gamma_2$, $\alpha_1\beta_2\gamma_2$(D75C) and α_1(K104C)$\beta_2\gamma_2$(D75C) receptors did not significantly differ from those of wildtype receptors (**Figure 14A**). However, one-way ANOVAs revealed that lower GABA concentrations (3μM and 10μM) produced greater responses in α_1(K104C)$\beta_2\gamma_2$(D75C) receptors compared to the single mutants and wildtype receptors (see **Figure 14** legend for statistics). Despite the model-based hypothesis that the electrostatic interaction between α_1K104 and γ_2D75 is predicted to occur after diazepam binding, substituting these residues with cysteines could allow a disulfide bond to form spontaneously, which would be able to form between residues at greater distances apart than an electrostatic bond. Therefore, we tested if the disulfide bond between α_1K104C and γ_2D75C had spontaneously occurred. The reducing agent dithiothreitol (DTT) is able to break accessible disulfide bonds. Application of 2mM DTT to the α_1(K104C)$\beta_2\gamma_2$(D75C) receptor resulted in an increase in the GABA EC_5, from 3.6 ± 0.4 μM before DTT application to 10.5 ± 0.35μM after DTT (**Figure 14B**). This is due to the breakage of a single inter-subunit disulfide bond as shown in **Figure 15A**.

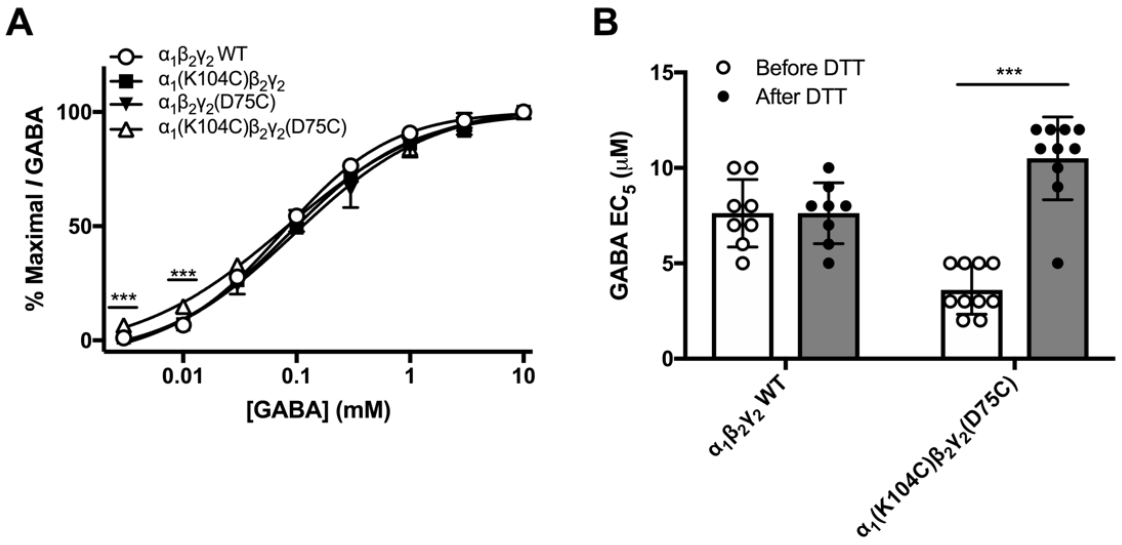

Figure 14. Formation and breakage of the disulfide bond between α_1(K104C) and γ_2(D75C) affects responses to GABA.

A. GABA concentration-response curves were generated in wildtype $\alpha_1\beta_2\gamma_2$, single mutant α_1(K104C)$\beta_2\gamma_2$ and $\alpha_1\beta_2\gamma_2$(D75C), and double mutant α_1(K104C)$\beta_2\gamma_2$(D75C) $GABA_A$ receptors. A repeated-measures ANOVA revealed no difference in the concentration-response curve between wildtype and mutant receptors. However, one-way ANOVAs showed significant effects of mutation at 3 μM [$F(3,26)=15.504$, $p<0.001$] and 10 μM GABA [$F(3,26)=18.163$, $p<0.001$], with a Tukey's post-hoc test at both concentrations showing a significant increase in response in α_1(K104C)$\beta_2\gamma_2$(D75C) receptors compared to the other three receptors (***, $p<0.001$). Some symbols are hidden behind other symbols. **B.** DTT (2 mM, dark symbols and bars) increased the absolute concentration of GABA required to produce an EC_5 response in α_1(K104C)$\beta_2\gamma_2$(D75C) but not wildtype receptors. A two-way ANOVA followed by a Tukey's post-hoc test revealed a significant effect of DTT treatment on α_1(K104C)$\beta_2\gamma_2$(D75C) receptors (***, $p<0.001$). Each symbol represents the GABA EC_5 of one oocyte, and each bar represents the mean GABA EC_5 ± SEM.

To further probe if α_1K104C and γ_2D75C spontaneously form a disulfide bond in the α_1(K104C)$\beta_2\gamma_2$(D75C) receptor, we tested PMTS for its effects. PMTS is able to covalently bind to free cysteine residues to which it has access. PMTS caused a significant decrease in GABA EC_5 current in the single and double mutant receptors (**Figure 15B**, hollow bars with open circles). In the wildtype and both single cysteine mutant receptors, the effect of PMTS remained unchanged after a prior DTT application (**Figure 15B**, hollow bars with triangles). This indicates that in single mutant receptors the cysteine substituted residues do not form disulfide bonds with endogenous cysteines in $GABA_A$ receptor. Since these single mutant and wildtype receptors exhibited similar changes in response to PMTS before and after DTT application, we did not test these receptors again 60 minutes after DTT treatment. For the α_1(K104C)$\beta_2\gamma_2$(D75C) receptor, a one-way ANOVA revealed a significant effect of PMTS treatment before, 5 minutes after, and 60 minutes after DTT treatment [$F(2,13)=108.363$, $p<0.001$]. Without prior exposure to DTT, application of PMTS resulted in a decrease in current (**Figure 15B**, white bar, open circles). However, DTT application before PMTS resulted in an increase in current (solid bar). Waiting 60 minutes after DTT washout, and then applying PMTS, resulted in a decrease in current similar to that seen with PMTS application before DTT application. For the double cysteine mutant receptor the white bar with open circles represents PMTS binding to the single available cysteine residue situated between the α and β subunit interfaces as shown in the illustration on the left in **Figure 15A**. When DTT breaks the sole disulfide bond between α and γ subunits, PMTS can now bind to up to three free cysteines. Since there was no significant difference between PMTS application before DTT application and 60 minutes after DTT application, we hypothesize that the disulfide bond breakage produced by DTT is only temporary, and that the receptor spontaneously returns to its pre-DTT form within an hour. The reformation of the disulfide bond in the double mutant receptor was also seen experimentally by repeatedly applying the GABA EC_5 to the DTT-treated

receptor and observing a gradual increase in current (**Figures 15C and 15D)**. The current produced by a maximally-effective concentration of GABA was not changed by applying DTT.

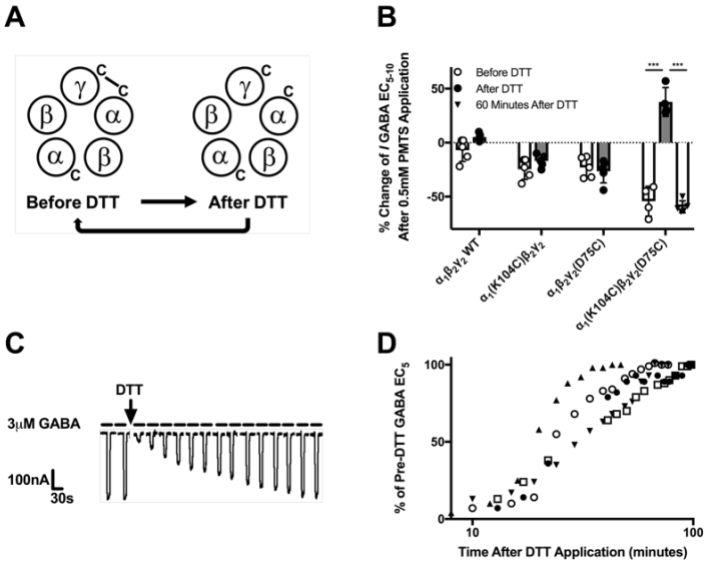

Figure 15. α_1(K104C)$\beta_2\gamma_2$(D75C) receptors spontaneously crosslink, and reform this crosslink after DTT application.

A. Illustration depicting the disulfide bond that spontaneously crosslinks between the α_1 and γ_2 subunits of the α_1(K104C)$\beta_2\gamma_2$(D75C) $GABA_A$ receptor, and that is broken after DTT application, slowly reforming over time. **B.** Effect of PMTS application on currents elicited by the GABA $EC_{5\text{-}10}$ of wildtype, α_1(K104C)$\beta_2\gamma_2$, $\alpha_1\beta_2\gamma_2$(D75C) and α_1(K104C)$\beta_2\gamma_2$(D75C) $GABA_A$ receptors. The change in GABA $EC_{5\text{-}10}$ currents by PMTS was decreased in single mutant receptors both before [$F(3,18) = 14.56$, $p < 0.05$] and after [$F(3,18) = 45.45$, $p < 0.001$] DTT application compared to those seen in wildtype receptors. The change in $EC_{5\text{-}10}$ currents produced by PMTS was not significantly altered after DTT application to wildtype or single mutant receptors, but did significantly change in double mutant receptors [$F(3,37)=41.698$, $p<0.001$)]. A one-way ANOVA showed a significant effect of time after DTT treatment (pre-DTT treatment, 5 minutes after and 60 minutes after) on α_1(K104C)$\beta_2\gamma_2$(D75C) $GABA_A$ receptor [$F(2,13)=108.363$, $p<0.001$], and a Tukey's post-hoc test showed a significant difference between pre-DTT and 5 minutes after DTT application and a significant difference between 5 minutes after DTT and 60 minutes after DTT (***, $p<0.001$; each symbol represents an oocyte, and bars represent the mean ± SEM). **C.** Sample tracing showing spontaneous re-formation of the α_1-γ_2 inter-subunit disulfide bond in the α_1(K104C)$\beta_2\gamma_2$(D75C) $GABA_A$ receptor. The GABA EC_5 measured in the oocyte before DTT application was 3μM GABA, but after DTT application 3μM GABA elicited a much smaller response. After approximately 60 minutes the response to 3μM GABA had returned to pre-DTT levels. **D.** Time courses of EC_5 values plotted for 5 oocytes expressing α_1(K104C)$\beta_2\gamma_2$(D75C) receptors returning to their pre-DTT values. This can also be interpreted as the time required to re-form the disulfide bond after DTT application. The average time to return to half of the pre-DTT EC_5 was 26.9 ± 2.5 minutes.

3.3.4 EFFECT OF CYSTEINE SUBSTITUTION AT α_1K104 AND γ_2D75 ON BENZODIAZEPINE-SITE RESPONSES

There were no effects of DTT application on the modulation produced by 1μM diazepam, flunitrazepam, Ro 15-4513 or zolpidem on wildtype receptors, as expected. This was also the case for α_1(K104C)$\beta_2\gamma_2$ and $\alpha_1\beta_2\gamma_2$(D75C) receptors. However, application of DTT to the α_1(K104C)$\beta_2\gamma_2$(D75C) receptor produced an increase in diazepam potentiation (from 76.6 ± 6.3% to 136.7 ± 9%) and flunitrazepam potentiation (from 121.2 ± 9.1% to 201 ± 22.3%), and a decrease in potentiation by Ro 15-4513 (from 1 ± 3.2 to -17.8 ± 1.9%) (**Figure 16A-C**). DTT treatment rescued responses of α_1(K104C)$\beta_2\gamma_2$(D75C) receptors to wildtype levels by flunitrazepam and Ro 15-4513, but not by diazepam. Application of the non-classical benzodiazepine, zolpidem, did not produce a significant interaction between receptor mutant and DTT treatment, but a Tukey's post-hoc test revealed a small significant difference between pre- and post-DTT treatment in α_1(K104C)$\beta_2\gamma_2$(D75C) receptors ($p<0.05$) (**Figure 16D)**. Interestingly, Ro 15-4513 produced a greater inhibitory response in the $\alpha_1\beta_2\gamma_2$(D75C) mutant compared to wildtype receptors, both before and after DTT treatment (**Figure 16C**). Treatment with 0.3% H_2O_2 for 90 seconds, which would favor cysteine bond reformation, before the benzodiazepine application reversed the effects of DTT in α_1(K104C)$\beta_2\gamma_2$(D75C) receptors, but produced no changes in responses by wildtype receptors (**Figures 17A** and **17B**).

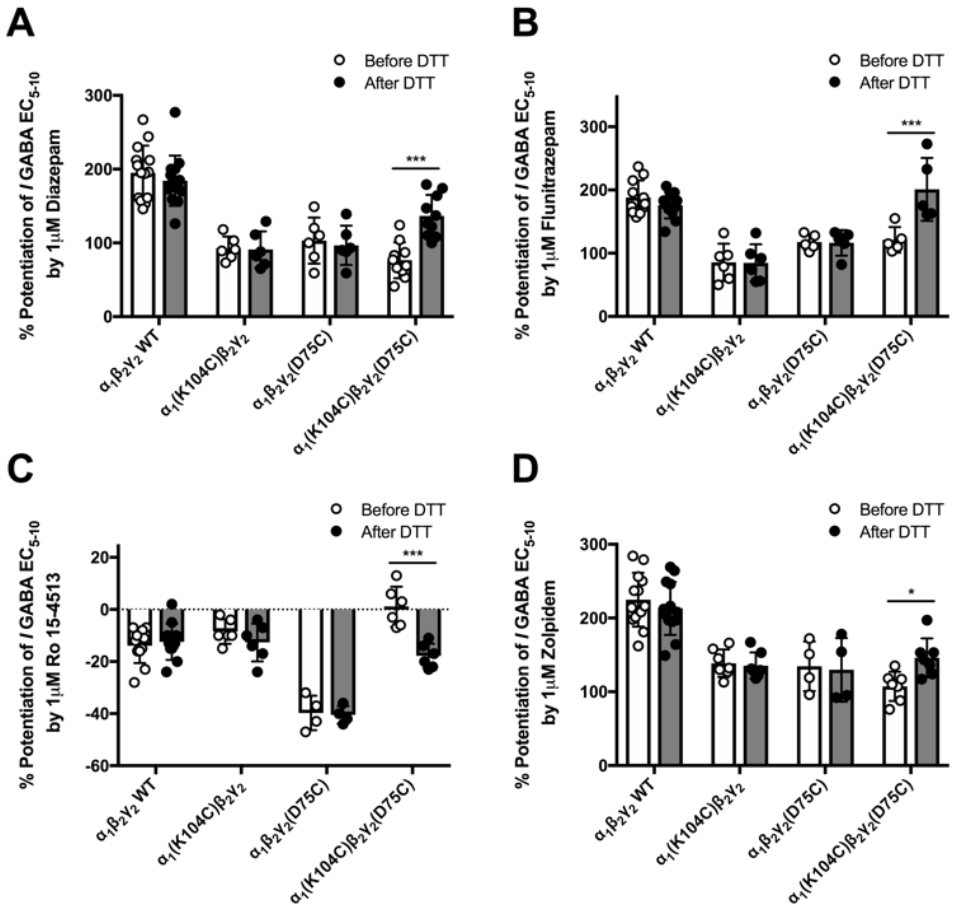

Figure 16. Benzodiazepine responses of wildtype and mutant $GABA_A$ receptors before (white symbols and bars) and after (dark symbols and bars) DTT application.

Bar graphs showing the percent potentiation of GABA EC_{5-10} in wildtype, $\alpha_1(K104C)\beta_2\gamma_2$, $\alpha_1\beta_2\gamma_2(D75C)$ and $\alpha_1(K104C)\beta_2\gamma_2(D75C)$ $GABA_A$ receptors produced by 1μM **(A)** diazepam, **(B)** flunitrazepam, **(C)** Ro 15-4513, and **(D)** zolpidem. A two-way ANOVA followed by a Tukey's multiple comparison post-hoc test showed a significant increase in all benzodiazepine-site responses after DTT application to $\alpha_1(K104C)\beta_2\gamma_2(D75C)$ $GABA_A$ receptors but not wildtype or single mutant receptors (*, $p<0.05$; ***, $p<0.001$, with each symbol representing the percent potentiation of the GABA EC_{5-10} seen in one oocyte, and each bar representing the mean percent potentiation ± SEM).

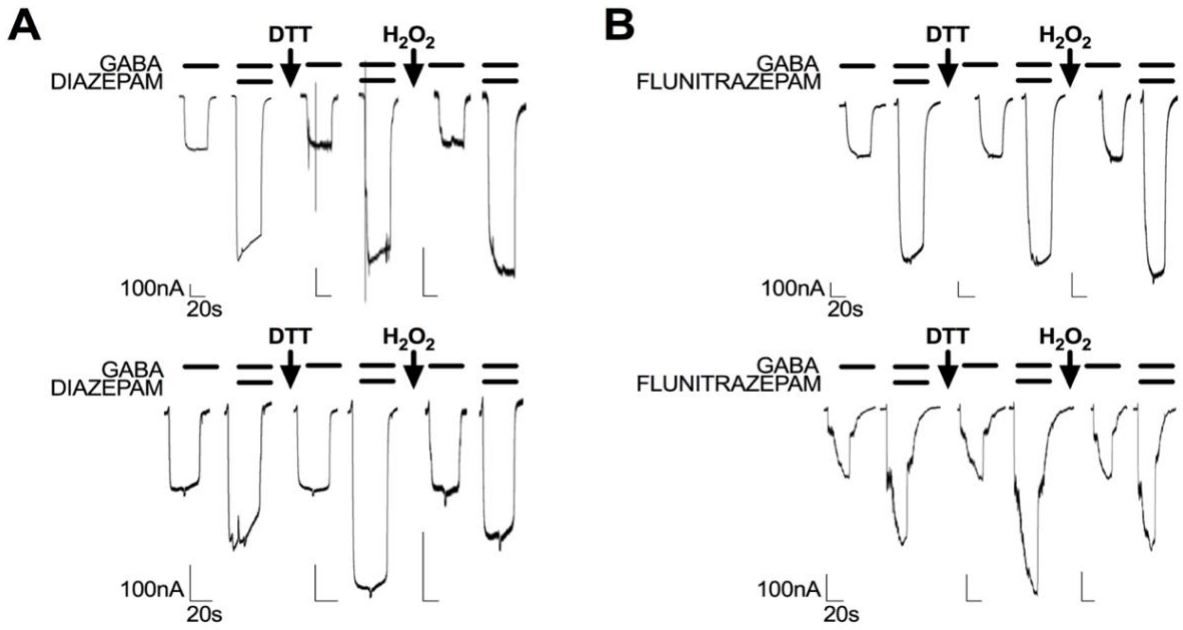

Figure 17. Sample tracings showing the effects of DTT and H_2O_2 treatment on potentiation by 1μM diazepam and 1μM flunitrazepam.

The top panels show tracings obtained from oocytes expressing wildtype receptors while the bottom panel shows tracings of oocytes expressing α_1(K104C)$\beta_2\gamma_2$(D75C) $GABA_A$ receptors. DTT application to α_1(K104C)$\beta_2\gamma_2$(D75C) receptors increased both diazepam **(A)** and flunitrazepam **(B)** potentiation, and hydrogen peroxide application reversed this increase back to pre-DTT levels.

3.3.5 EFFECTS OF CYSTEINE SUBSTITUTION ON NON-BENZODIAZEPINE MODULATORS OF THE $GABA_A$ RECEPTOR

Allosteric modulators of the $GABA_A$ receptor acting at sites other than the benzodiazepine binding site were next tested to determine the specificity of the electrostatic interactions between α_1(K104C) and γ_2(D75C). Ethanol and the neurosteroid allopregnanolone produced similar potentiation of the effects of GABA on wildtype, α_1(K104C)$\beta_2\gamma_2$, $\alpha_1\beta_2\gamma_2$(D75C) and α_1(K104C)$\beta_2\gamma_2$(D75C) $GABA_A$ receptors (**Figure 18**). There was no significant effect of DTT treatment on the enhancement of wildtype or mutant receptors by 200mM ethanol, 100nM allopregnanolone (**Figure 18**) or 1μM allopregnanolone (data not shown).

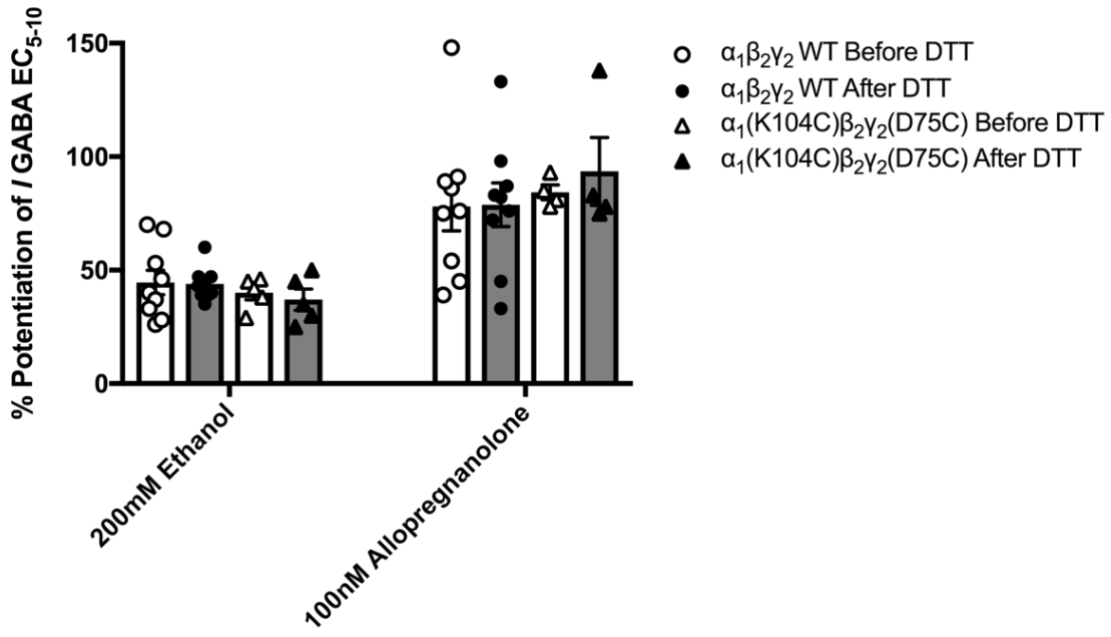

Figure 18. Modulators acting at sites other than the benzodiazepine site at wildtype and cysteine substituted $GABA_A$ receptors are unaffected by DTT treatment.

Before modulator effects were tested, the EC_{5-10} concentration of GABA was determined in each oocyte. Effects of 200mM ethanol and 100nM allopregnanolone were measured in wildtype and $\alpha_1(K104C)\beta_2\gamma_2(D75C)$ $GABA_A$ receptors before (white symbols and bars) and after (dark symbols and bars) application of DTT, with no significant changes being observed (Two-way ANOVA's: 200mM ethanol [$F(3,43)=0.031$]; 100nM Allopregnanolone [$F(3,45)=0.176$]. Each symbol represents the percent potentiation of the GABA EC_{5-10} seen in one oocyte, and each bar represents the mean percent potentiation ± SEM.

3.3.6 EFFECTS OF ALANINE SUBSTITUTION AT α_1K104 AND γ_2D75 ON GABA AND BENZODIAZEPINE RESPONSES

To examine the effects of alanine substitutions at the α_1K104 and/or γ_2D75 residues, we compared receptors containing these alanine residues to wildtype receptors in their responses to GABA, 1μM diazepam and 1μM flunitrazepam. The GABA concentration-response curve for $\alpha_1\beta_2\gamma_2$(D75A) was slightly right shifted (EC_{50} 133.6 ± 19.4 μM), while that of the α_1(K104A)$\beta_2\gamma_2$ receptor was slightly left shifted (EC_{50} 61.9 ± 12.2 μM) compared to the wildtype receptor curve (EC_{50} 77 ± 8.8 μM) (**Figure 19A**). A repeated-measures ANOVA revealed a significant difference among the four concentration-response curves (see **Figure 19A** legend for statistics). Interestingly, the α_1(K104A)$\beta_2\gamma_2$(D75A) GABA concentration-response curve (EC_{50} 87.3 ± 12.4 μM) was not left-shifted at lower concentrations (3μM and 10μM), unlike the α_1(K104C)$\beta_2\gamma_2$(D75C) receptor (**Figure 19A** compared to **Figure 14A**). One-way ANOVAs revealed that there was a significant effect of alanine substitution on the enhancement of GABA $EC_{5\text{-}10}$ by 1μM diazepam or flunitrazepam. While the single substitution α_1(K104A)$\beta_2\gamma_2$ and $\alpha_1\beta_2\gamma_2$(D75A) receptors exhibited a decreased response to diazepam and flunitrazepam compared to wildtype receptors, the α_1(K104A)$\beta_2\gamma_2$(D75A) receptors displayed a level of potentiation not significantly different from that of wildtype receptors (**Figure 19B**).

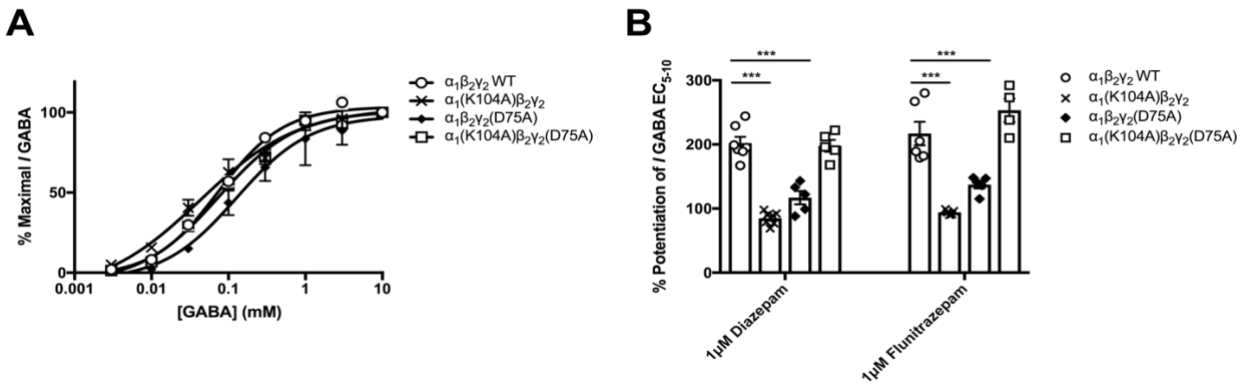

Figure 19. Effect of alanine substitution at α_1K104 and γ_2D75 on GABA sensitivity and benzodiazepine responses.

A. GABA concentration-response curves of wildtype, α_1(K104A)$\beta_2\gamma_2$, $\alpha_1\beta_2\gamma_2$(D75A), and α_1(K104A)$\beta_2\gamma_2$(D75A) receptors. The concentration-response curves were significantly different [$F(21,132)=1.937$, $p<0.05$]. Each symbol represents the data from 3-6 oocytes ± SEM. In some cases error bars fall within symbols. **B.** Bar graph comparing levels of diazepam and flunitrazepam potentiation between wildtype and alanine substituted receptors. Potentiation of GABA $EC_{5\text{-}10}$ by 1μM diazepam and 1μM flunitrazepam was decreased for single but not double alanine substitution mutants compared to wildtype receptors. A one way ANOVA revealed a significant effect of mutant on receptor potentiation by diazepam [$F(3,23)=51.407$, $p<0.001$] and flunitrazepam [$F(3,19)=26.926$, $p<0.001$]. Each symbol represents the percent potentiation observed in one oocyte, and each bar represents the mean percent potentiation ± SEM.

3.3.7 EFFECTS OF CHARGE REVERSAL OF α_1K104 AND γ_2D75 RESIDUES ON GABA AND GABA RECEPTOR MODULATOR RESPONSES

To test if reversing the charges of α_1K104 and γ_2D75 would restore GABA sensitivity, GABA concentration-response curves of α_1(K104D)$\beta_2\gamma_2$(D75K) were compared to those of wildtype receptors (**Figure 20A**). A repeated-measures ANOVA found a significant difference between wildtype and α_1(K104D)$\beta_2\gamma_2$(D75K) concentration response curves (see **Figure 20A** legend for statistics). The average EC_{50} value for wildtype receptors was 86.8 ± 16.5 μM while the EC_{50} for α_1(K104D)$\beta_2\gamma_2$(D75K) was increased to 146.3 ± 23.1 μM. The charge reversal did not restore to wildtype levels receptor potentiation by 1 μM diazepam or Ro 15-4513, but did restore potentiation by 1 μM flunitrazepam and zolpidem (**Figure 20B**). Other $GABA_A$ receptor modulators (200 mM ethanol and 100 nM allopregnanolone) displayed no changes in potentiation of GABA EC_{5-10} after charge reversal, compared to wildtype receptors (data not shown).

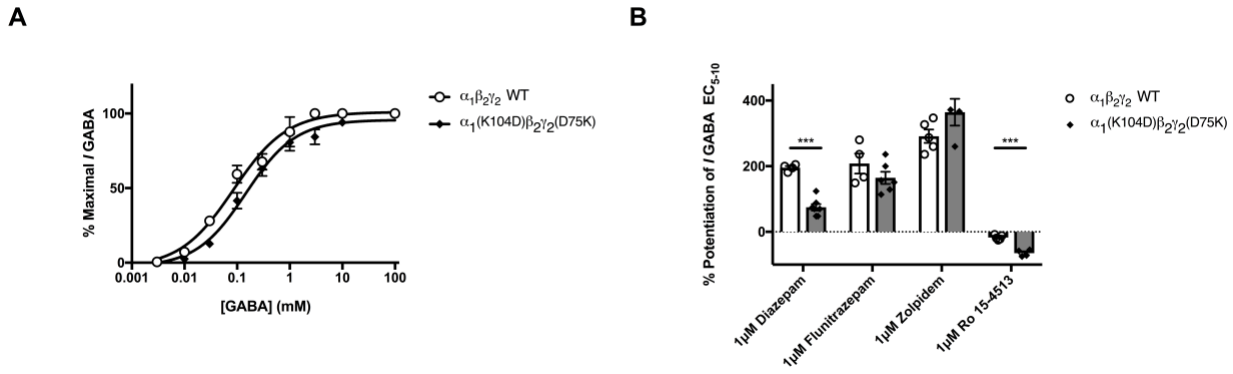

Figure 20. Charge reversal at α_1K104 and γ_2D75 does not rescue GABA sensitivity or benzodiazepine responses to wildtype responses.

A. The $\alpha_1(K104D)\beta_2\gamma_2(D75K)$ receptor GABA concentration-response curve is significantly right-shifted compared to wildtype receptors [$F(8,105)=2.8$, $p<0.01$]. The EC_{50} for wildtype receptors was 86.8 ± 16.5μM, increasing to 146.3 ± 23.1μM for the $\alpha_1(K104D)\beta_2\gamma_2(D75K)$ $GABA_A$ receptor. Each symbol represents the mean ± SEM from 5-6 oocytes. **B.** Bar graph comparing levels of benzodiazepine enhancement between $\alpha_1(K104D)\beta_2\gamma_2(D75K)$ and wildtype receptors. The $\alpha_1(K104D)\beta_2\gamma_2(D75K)$ $GABA_A$ receptor was unable to fully rescue responses to wildtype levels of potentiation by 1μM diazepam and Ro 15-4513, but was able to rescue the responses to 1μM flunitrazepam and zolpidem. Each symbol represents the percent potentiation of the GABA $EC_{5\text{-}10}$ seen in one oocyte, and each bar represents the mean potentiation ± SEM.

3.4 Discussion

Signal transduction of ligand-gated ion channels after neurotransmitter binding to its orthosteric site is believed to involve a wave of structural rearrangements in the receptor, and this rearrangement is thought to be separate from the signal transduction pathway produced by allosteric modulators (Grosman et al., 2000; Venkatachalan and Czajkowski, 2012). Using molecular modeling to identify potential electrostatic interactions between the α_1 and γ_2 subunits, we identified an interaction between α_1K104 and γ_2D75 that occurs after diazepam binding (**Figure 13**). It is likely that these residues interact to stabilize the positively-modified state of the receptor, and that this interaction is specific for the benzodiazepine signal transduction pathway.

Low concentrations of GABA produced greater responses in α_1(K104C)$\beta_2\gamma_2$(D75C) than in wildtype receptors, but this was not seen at higher GABA concentrations (**Figure 14A**). This increased response at low GABA concentrations is similar to what one would expect to see in response to co-application of GABA with a benzodiazepine in wildtype receptors. Benzodiazepine site agonists increase the effects of low but not higher concentrations of GABA, since the ion channel approaches its maximal open probability at saturating GABA concentrations (Newland et al., 1991).

One would predict that a receptor that is behaving as though a benzodiazepine molecule has already bound would exhibit a decreased response to a co-application of benzodiazepine with GABA. In the α_1(K104C)$\beta_2\gamma_2$(D75C) receptor, the two cysteine residues spontaneously formed a disulfide bond. Accordingly, we saw the expected decrease in diazepam, flunitrazepam, and zolpidem potentiation in the double cysteine substituted receptors (**Figures 16A, 16B and 16D**). After the disulfide bond is broken with DTT, responses to these benzodiazepines increase, suggesting that an electrostatic bond between these residues in wildtype receptors formed in

response to benzodiazepine binding. After DTT application, the response of α_1(K104C)$\beta_2\gamma_2$(D75C) receptors to flunitrazepam potentiation was rescued to wildtype levels (**Figure 16B**). One reason why potentiation by flunitrazepam, but not diazepam or zolpidem, may be completely rescued following DTT application is that the latter two show weaker modulatory responses after α_1K104C-γ_2D75C disulfide bond formation than flunitrazepam; i.e., lower potentiation of GABA responses before DTT application. The conformational rearrangement within the $GABA_A$ receptor after benzodiazepine binding most likely depends on the formation of multiple bonds, not just α_1K104-γ_2D75. Thus, the α_1K104 - γ_2D75 bond may be less important for flunitrazepam potentiation than for diazepam or zolpidem.

Zolpidem is a non-classical benzodiazepine and at low concentrations is selective for the α_1 subunit-containing $GABA_A$ receptor over those containing other α subunits (Langer et al., 1992). Disulfide trapping within the γ_2 subunit has shown that the conformational change produced by classical benzodiazepines may not be the same as that produced by zolpidem (Hanson and Czajkowski, 2011). Similarly, there are mutations in the α_1 and γ_2 subunits that affect classical but not non-classical benzodiazepines, or vice versa (Mihic et al., 1994; Buhr and Sigel, 1997; Bowser et al., 2002; Hanson and Czajkowski, 2008). The magnitude of the increase in α_1(K104C)$\beta_2\gamma_2$(D75C) receptor potentiation by zolpidem after DTT application was far smaller than the increase seen with the classical benzodiazepines diazepam and flunitrazepam (**Figure 16**). We speculate that this may be due to classical and non-classical benzodiazepines producing overlapping yet distinct conformational changes in the $GABA_A$ receptor after binding.

One might hypothesize that the conformational changes produced by benzodiazepines would be different than those produced by inverse agonists such as Ro 15-4513. Indeed, previous studies have shown that this might be the case, where disulfide trapping at the α-γ interface of the

$GABA_A$ receptor, that affected benzodiazepine potentiation, had no effect on inverse benzodiazepine inhibition (Hanson et al., 2011). Our work supports this hypothesis, as the α_1(K104C)$\beta_2\gamma_2$(D75C) mutant $GABA_A$ receptor, which traps the receptor in a 'positively modified' state, was not inhibited by Ro 15-4513 as much as wildtype receptors (**Figure 16C**). Once DTT is applied, the α_1(K104C)$\beta_2\gamma_2$(D75C) receptor is relieved of this 'positively modified state', and Ro 15-4513 is able to produce inhibition to levels similar to that of wildtype receptors (**Figure 16C**). Ro 15-4513 produced more inhibition in the $\alpha_1\beta_2\gamma_2$(D75C) receptor than in the wildtype, α_1(K104C)$\beta_2\gamma_2$, and α_1(K104C)$\beta_2\gamma_2$(D75C) receptors. Substituting the γ_2D75 residue with a lysine or alanine residue increased the inhibition by Ro 15-4513 even more so than the cysteine replacement at that residue (data not shown). This decrease suggests that the γ_2D75 residue may be involved in the conformational change produced by Ro 15-4513 as well as a distinct conformational change produced by potentiating benzodiazepines.

After DTT breaks the disulfide bond in α_1(K104C)$\beta_2\gamma_2$(D75C) receptors, one might expect the receptor to behave similarly to the α_1(K104A)$\beta_2\gamma_2$(D75A) receptor. While the α_1(K104A)$\beta_2\gamma_2$(D75A) receptor exhibited levels of diazepam and flunitrazepam potentiation similar to wildtype receptors (**Figure 19B**), DTT treatment to α_1(K104C)$\beta_2\gamma_2$(D75C) receptors did not fully restore levels of diazepam potentiation (**Figure 16A**). One possible explanation for this is that DTT breaking the disulfide bond by reducing each mutant cysteine) results in two hydrogen-bound cysteine residues that would occupy more volume than alanine residues at those positions, preventing the conformational change produced by diazepam from occurring. Another possibility is that in the α_1(K104C)$\beta_2\gamma_2$(D75C) receptor, the spontaneous reformation of a disulfide bond after DTT treatment (**Figure 15C and 15D**) prevents one from experimentally capturing the maximal amount of enhancement produced by diazepam.

The α_1(K104D)$\beta_2\gamma_2$(D75K) receptor, bearing two charge-reversing substitutions, displayed a right-shifted GABA concentration-response curve compared to wildtype receptors (**Figure 20A**). Additionally, the α_1(K104D)$\beta_2\gamma_2$(D75K) receptor did not restore GABA, diazepam, or Ro 15-4513 sensitivity to wildtype levels (**Figure 20B**). This is likely because the α_1K104 and γ_2D75 residues lie within a pocket of charges, and that modifying these residues is preventing other interactions from occurring; i.e., although K104D and D75K substitutions may restore the electrostatic interaction between these residues, there are other charged residues near these sites that may now interact differently with the reversed charge residues, compared to the original wildtype amino acids. Evidently, the α_1K104 - γ_2D75 interaction is not the only interaction that is important for producing the positively modified state of the receptor. If it were, one would see no benzodiazepine potentiation of the receptor after mutating the α_1K104 and γ_2D75 residues.

One might argue that the data obtained from the alanine substitution experiments at α_1K104 and γ_2D75 do not fit our overall hypothesis that formation of a bond between these two residues facilitates benzodiazepine effects at the $GABA_A$ receptor. Perhaps what is happening is that during the conformational changes produced by benzodiazepine site agonists at wildtype receptors these two charged residues come close enough together to at least partially neutralize each others' charges. This hypothesis is supported by results obtained using the single alanine substitutions, which retain single charged residues in each pair, and display weaker effects of benzodiazepines than those seen in the double alanine mutant (**Figure 19B**). A possible explanation may be that the retained charged residue in the single mutants may still be interacting with other nearby charged residues (egs. α_1K105, γ_2D148 or γ_2R197), thus retarding the ability of the receptor to adopt the benzodiazepine-activated conformational state. This would also apply to the single cysteine substitutions which also display decreased responses to diazepam and flunitrazepam. In scenarios in which the $\alpha_1$104 and $\gamma_2$75 residues are in close proximity (eg.,

cysteines crosslinked) the receptor has already adopted a benzodiazepine positively-modified state and thus adding exogenous benzodiazepine does not have much effect. In cases where these two residues are not initially close together, but are capable of moving closer together, (e.g. the double alanine substitutions or the double uncrosslinked cysteines) a greater effect of applied benzodiazepine will be seen. Lastly, in scenarios in which one or the other of these residues is constrained in its movement (e.g. single substitutions), benzodiazepine effects would be smaller due to the remaining charged residue.

An initial concern was that the receptor mutants were not being incorporated correctly on cell surfaces and that oocytes were expressing primarily $\alpha_1\beta_2$ receptors, not $\alpha_1\beta_2\gamma_2$ receptors. Previous studies used $ZnCl_2$ to test for γ_2 subunit incorporation, as zinc inhibits $\alpha_1\beta_2$ receptors to a greater extent than $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors (Draguhn et al., 1990). However, using this test may not be the most accurate way to test for $\alpha\beta$ contamination, as even a small fraction of $\alpha_1\beta_2$ receptors present may produce a significant inhibitory effect by zinc (Boileau et al., 2002). Interestingly, the $\alpha_1\beta_2$ receptors display an increase in their GABA-evoked currents after DTT treatment, but $\alpha_1\beta_2\gamma_2$ receptor currents are unchanged (Amato et al., 1999). In our study, we saw no change in GABA-evoked currents after DTT treatment in wildtype and mutant receptors, except for α_1(K104C)$\beta_2\gamma_2$(D75C) receptors, where we actually saw a decrease in GABA-evoked currents (**Figure 15D**). This, together with the fact that we injected receptors cDNAs in a $1\alpha_1$:$1\beta_2$:$10\gamma_2$ cDNA ratio and we still saw a benzodiazepine effect, engenders confidence that the receptors are incorporating wildtype and mutated γ_2 subunits.

One interesting and clinically-relevant aspect of this study revolves around the additive and synergistic properties of $GABA_A$ receptor modulators. Benzodiazepines are often co-abused with ethanol, and the two classes of compounds are thought to act additively or synergistically as

central nervous system depressants (*Dawn Report*, 2014). While ethanol is thought to act at the α+/β- interface in αβδ $GABA_A$ receptors, it is not clear if this is necessarily the case in αβγ receptors (Wallner et al., 2014). We tested if mutations that affect both GABA and benzodiazepine responses also produced changes in ethanol responses. In the α_1(K104C)$\beta_2\gamma_2$(D75C) 'positively modified' receptor, no changes in ethanol potentiation were observed (**Figure 18**). Similarly, the charge reversal α_1(K104D)$\beta_2\gamma_2$(D75K) receptor exhibits similar ethanol potentiation to that of wildtype receptors (data not shown). These data suggest that the conformational changes in the $GABA_A$ receptor produced by ethanol are experimentally separable from the conformational changes produced by benzodiazepines, and that both can occur simultaneously to further enhance receptor function. This provides a possible molecular mechanism for the synergistic/additive effects of benzodiazepines and alcohol.

The neurosteroid allopregnanolone acts as a potent modulator of the $GABA_A$ receptor, as well as a direct activator at high concentrations. The binding site for this enhancing action is thought to be within a cavity formed by transmembrane domains one and four within a single α subunit (Akk et al., 2007; Hosie et al., 2006). The α_1(K104C)$\beta_2\gamma_2$(D75C) 'positively modified' receptor and α_1(K104D)$\beta_2\gamma_2$(D75K) charge reversal receptor displayed no differences in their sensitivities to the potentiating effects of allopregnanolone, compared to wildtype receptors (**Figure 18**). As well as having distinct binding sites, our data suggest that allopregnanolone and benzodiazepines produce distinct conformational changes in the $GABA_A$ receptor.

In summary, our study suggests that an inter-subunit electrostatic interaction between α_1K104 and γ_2D75 occurs after benzodiazepine site agonist binding to help stabilize the $GABA_A$ receptor in a positively modified state. This interaction seems to be more important for classical (non-selective between $GABA_A$ receptor α subunits) benzodiazepines than non-classical (α_1

selective) compounds. Additionally, this interaction does not seem to be important for modulators of the $GABA_A$ receptor acting at non-benzodiazepine sites, suggesting that the α_1K104-γ_2D75 interaction is specific for benzodiazepine site agents.

CHAPTER 4: USE OF PHAGE DISPLAY TO IDENTIFY NOVEL COMPOUNDS THAT ACT ON THE $GABA_A$ RECEPTOR

4.1 Introduction

Despite animal knockout data suggesting that α_2-containing $GABA_A$ receptors are a therapeutic target for anxiety, no α_2-specific $GABA_A$ receptor modulator or agonist is currently on the market for the treatment of anxiety (Tan et al., 2011). This chapter details how phage display can be employed as a technique to find compounds specific for the $\alpha_2\beta_3\gamma_2$ receptor. We utilize bacteriophage which express a foreign peptide, seven amino acids long, as part of one of its coat proteins. The peptide-expressing bacteriophage is 'panned' against a target to which the peptide could bind, in our case Human Embryonic Kidney-293 (HEK-293) cells expressing the $GABA_A$ receptor. This technique has advantages over other methods of drug discovery, such as synthetic small molecule libraries, since up to 10^{10} unique peptides can be screened at one time, compared to ~50,000 small compounds (Inglese et al., 2007, Molek et al., 2011).

This technique has proven to be a successful method of drug discovery, with five drugs derived via phage display currently approved by the Food and Drug Administration, such as the anti-inflammatory agent Humira®. Many other drugs derived through phage display are currently in clinical trials, for indications ranging from cancer to Alzheimer's disease (Nixon et al., 2014). Our lab was the first to use this technique to identify peptide allosteric modulators for a ligand-gated ion channel, specifically the ionotropic glycine receptor. Further, peptides that act on the glycine receptor with subunit selectivity were obtained by panning against unwanted subunit compositions (negative selection), before panning against the desired composition (positive selection) (Cornelison et al., 2016). This success, coupled with the fact that glycine and $GABA_A$

receptors are both members of the same receptor superfamily, increases the likelihood that phage display could identify selective peptide modulators of the $\alpha_2\beta_2\gamma_2$ $GABA_A$ receptor.

4.2 Methods

All products were purchased from Thermo Fisher Scientific, Waltham, MA, unless otherwise noted.

4.2.1 CELL CULTURE

Human Embryonic Kidney (HEK) 293 cells were obtained from American Type Culture Collection (Manassas, VA) and stored in liquid nitrogen before use. Prior to beginning phage display, cells were thawed and placed in four wells of a polystyrene 12-well tissue culture plate (Celltreat, Pepperell, MA), with each well containing 2ml of Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich, St. Louis, MO) supplemented with 1% of 100x Glutamax, 10% fetal bovine serum, 1% 100x MEM non-essential amino acid solution and 1% penicillin streptomycin (5000U/ml penicillin and 5000μg/ml streptomycin) (Invitrogen, Carlsbad, CA). These cells were incubated at 37ºC and 5% CO_2. Every three days the cells were split by aspirating the DMEM from the cells and rocking the cells in 2ml of 0.25% Trypsin-EDTA solution for two minutes to detach the cells from each well. The detached cells were placed in new wells containing fresh DMEM in a 1:5 ratio. After five rounds of cell passaging, and when cells achieved around 50% confluency, one well containing cells was transfected with 1μg $\alpha_2\beta_3\gamma_2$ receptor DNA (in a $1\alpha_2$:$1\beta_3$:$10\gamma_2$ ratio) using a Polyfect kit (Qiagen, Valenica, CA), while the other three wells were left un-transfected. The cells were incubated for 24-36 hours before the phage display panning began.

4.2.2 PHAGE DISPLAY PANNING

The Ph.D.7 phage display library, consisting of phage expressing heptapeptides, was purchased from New England Biolabs (Ipswich, MA). Two wells containing un-transfected HEK-293 cells were washed three times with phosphate buffered saline supplemented with 1.5% bovine serum albumin and 0.1% Tween-20 (PBS+BSA/Tween). 4µl of the phage supplied in the kit (2 x 10^{11} phage particles) was diluted in 366µl of PBS+BSA/Tween and applied to two of the wells and rocked at room temperature. After 30 minutes, the unbound phage from one of these wells was applied to another un-transfected well, while the unbound phage from the other well was applied to the $\alpha_2\beta_3\gamma_2$ transfected well and rocked at room temperature. After 60 minutes, the unbound phage from the second set of wells were removed and the cells in these wells were washed five times with PBS+BSA/Tween. To elute the phage, 366µl of 0.2M glycine hydrochloride (supplemented with 1mg/ml bovine serum albumin) was added to the second set of wells, and the cells were rocked for 10 minutes at room temperature.

After 10 minutes, the 366µl eluate was neutralized with 55µl of 1M tris-hydrochloride (pH 9), and the solution was removed from the well. 10µl of this solution was saved for tittering, while the rest of the solution was used for the amplification of phage.

4.2.3 AMPLIFICATION OF PHAGE

The neutralized eluate from the phage display panning described above was added to a flask containing an overnight (A600) *Escherichia coli* culture in 20ml Luria broth (LB), and shaken at 250rpm for 4.5 hours. Cells were then spun down for 10 minutes at 4000g at 4ºC, after which 16ml of the supernatant was removed, added to 4ml of Polyethlylene glycol 8000(20%)/NaCl (PEG/NaCl), and incubated on ice overnight. The next day the 20ml sample was

centrifuged at 12,000g for 10 minutes at 4ºC, after which the supernatant was removed and the pellet re-suspended in 1ml tris-buffered saline. Following a 10-minute incubation at room temperature, the 1ml solution was spun for one minute at 12,000g, and the resulting liquid transferred to a clean tube. PEG/NaCl (1/6 volume) was added to re-precipitate the phage, and the mixture incubated on ice for 15 minutes before being spun for 10 minutes at 12,000g. The supernatant was then removed, and the phage re-suspended in 200µl tris-buffered saline. 10µl of the amplified phage was saved for titering, and the remainder saved for the subsequent phage panning round.

4.2.4 PHAGE TITERING

Phage titering was essential for two components of the phage display process; first, to determine output of phage from each round to observe selection for the target, and second, to measure the amplified amount of phage so that the necessary amount could be applied in the subsequent panning round.

Prior to phage display titering, LB/IPTG/XGAL (per 1 liter LB: 15g agar, 0.05g isopropyl-β-D-thiogalactoside (IPTG), 0.04g 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (XGAL), 1mL dimethyl formamide) plates were made and stored for up to one week at 4ºC. These plates were warmed prior to phage titering. The library cloning vector M13KE has a gene inserted in it coding for LacZα, which results in blue phage plaques on LB/IPTG/XGAL plates. Additionally, a 5ml culture of *Escherichia coli* ER2738 was shaken at 250rpm and 37ºC for approximately 5 hours, until cells were in mid-log phase, and then 200µl of this culture was each placed into 8 Eppendorf tubes.

24ml of top agar (per 1 liter: 10g Bacto-Tryptone, 5g yeast extract, 5g NaCl, 7g Bacto-Agar) was melted and aliquoted amongst eight polystyrene tubes. Serial 10-fold dilutions were prepared of both the phage eluate (10^2-10^5 final dilution) and the amplified phage (10^8-10^{11}final dilution). 10µl of each phage dilution was then added to Eppendorf tubes containing 200µl of the mid-log phase ER2738 cells, vortexed, incubated for two minutes at room temperature, then added to a polystyrene tube containing top agar. The top agar-infected cell mixture was immediately vortexed and spread onto LB/IPTG/XGAL plates, which were then inverted and incubated overnight at 37ºC. Plates that contained 40-100 plaques were counted and multiplied by their dilution factor to determine phage output from the previous round and phage input for the subsequent round.

4.2.5 PHAGE SEQUENCING

After three rounds of panning, individual blue plaques were picked and placed in 1ml of LB, incubated at 37ºC and shaken at 250rpm overnight. The following day the culture was purified using an Invitrogen mini-prep kit according to manufacturer's instructions, and phage DNA was sequenced at the University of Texas at Austin sequencing core using a 96III sequencing primer. This was repeated after rounds 4 and 5, and output sequences were analyzed for possible consensus sequences and traits. Peptides were ordered from and synthesized by Genscript with N-terminal acetylation.

4.2.6 PEPTIDE SCREENING WITH 2-ELECTRODE VOLTAGE CLAMP ELECTROPHYSIOLOGY

Xenopus laevis oocytes were harvested and isolated as described in chapter two, and injected with 1.5ng/30nl of either $GABA_A$ ($\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_3\gamma_2$, $\alpha_2\beta_3$; in 1:1:10, 1:1:10 or 1:1 ratios, respectively) or glycine (α_1 or α_1 W170S) receptor DNA using a Nanoject II (Drummond

Scientific Co., PA). Oocytes expressing $GABA_A$ receptor DNA were clamped at -80mV and perfused with ND-96 buffer while oocytes expressing glycine receptor DNA were voltage clamped at -70mV and perfused with Modified Barth's Saline, both as described in chapter two.

Once voltage clamped, the receptor agonist EC_5 (agonist concentration which produced a 5% maximal effect) of GABA or glycine receptors was determined and repeated at four minute intervals until responses were stable and did not vary by more than 5%. To test for peptide function at glycine or GABA receptors, 100μM of each peptide was pre-applied for 30 seconds, and then co-applied with agonist EC_5. This was followed by another agonist EC_5 application, to account for possible current drift. This experiment was repeated for the $GABA_A$ $\alpha_2\beta_3$ receptor and the glycine α_1 receptor in the presence of the zinc chelator tricine to control for possible zinc contamination in the peptides. The percent modulation produced by peptides was measured as $[(I_{EC5+\ peptide}/I_{EC5})-1] *100$.

4.3 Results

4.3.1 IDENTIFICATION OF PEPTIDES FROM PHAGE DISPLAY PANNING

Peptides identified from rounds 3-5 were divided into two groups. The first group consisted of peptides that appeared in both the control experiment (phage panned twice against un-transfected HEK-293 cells) and in the $\alpha_2\beta_3\gamma_2$ experiment (phage panned once against un-transfected HEK-293 cells and then again against $\alpha_2\beta_3\gamma_2$ transfected HEK-293 cells). 23 peptides were identified that fit these criteria (**Table 1**, left panel). Several traits were identified amongst peptides that fell in this group. Serine was the first amino acid in 7/23 of the peptides, and the second amino acid in 6/23 peptides. Proline appeared in position 4 of the heptapeptide in 5/23, and in position 7 5/23 times. The second group of peptides were peptides that were found in the $\alpha_2\beta_3\gamma_2$

experimental group (phage panned once against un-transfected HEK-293 cells then against $\alpha_2\beta_3\gamma_2$ transfected HEK-293 cells), but not in the control experiment (phage panned twice against un-transfected HEK cells). 11 peptides fit into this category (**Table 1**, right panel). In contrast to group 1, only 2 of 11 peptides in group 2 had a serine in position 1, and 0 peptides had a serine in position 2. Proline did appear in position 4 twice in group 2, but only once in position 7. Positively charged amino acids in two consecutive positions were observed in 3/23 (13%) heptapeptides in group 1, and these occurred between positions 2 and 5 of the peptide. In contrast, 3/11 (27%) of peptides from experimental group 2 had two positive amino acids in two consecutive positions, and these occurred between position 5 and 7. Two of these peptides from group two had the motif proline-x-arginine-histidine, where x was either tyrosine or glutamine. This difference in positive amino acid residue placement appeared to be the main difference between the two groups of peptides, and so the three peptides in group 2 that had two positive amino acids in consecutive positions were synthesized and probed electrophysiologically for $GABA_A$ receptor activity.

PEPTIDES APPEARING IN CONTROL AND $\alpha_2\beta_3\gamma_2$ GROUPS	
NSMKHVH	QNNIHTP
VLNSPNR	SSSYNSA
STTGTQY	YSEPAGG
MTPTKMP	QSVPRFH
NPSREYT	SSYIDYR
WLLPFRP	VHASLPS
SLTSDQD	SDFMQWD
ILMPTWP	FTPDGAR
SKHLSGF	EWHHKYT
QVNNLGE	VGLPMKP
SSWTGVV	GTIYWNS

PEPTIDES APPEARING ONLY IN $\alpha_2\beta_3\gamma_2$ GROUP
YMNPLEL
GLLSRTT
TMQTPTW
YQKWPAP
SWTVWRS
HFNPYRH
IHQKNFL
WVPQRHQ
TVQHLHR
SMNGVQV
DFRFTQS

Table 1: Sequences of heptapeptides identified through panning against non-transfected and $\alpha_2\beta_3\gamma_2$ transfected HEK-293 cells.

Left: 23 peptides identified from panning rounds 3-5 that appeared in both the control group (panned twice against non-transfected HEK-293 cells) and the $\alpha_2\beta_3\gamma_2$ group (panned against non-transfected HEK-293 cells followed by $\alpha_2\beta_3\gamma_2$ transfected HEK-293 cells). Right: 11 peptides were identified that appeared in the $\alpha_2\beta_3\gamma_2$ group but that did not appear in the control group. Highlighted in red in the right panel is the presence of positively charged amino acids found in two consecutive positions within the last three amino acids of the heptapeptide. This was not found in any of the peptides identified in the left panel.

4.3.2 PEPTIDE HFNPYRH HAS ACTIONS ON GABA$_A$ AND GLYCINE RECEPTORS

100μM HFNPYRH had inhibitory actions at $\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_3\gamma_2$ and $\alpha_2\beta_3$ GABA$_A$ receptors, with the most inhibition seen at $\alpha_2\beta_3$ receptors (**Figure 21A**, solid black bar). The addition of tricine (**Figure 21A**, solid gray bar) decreased inhibition of $\alpha_2\beta_3$ receptors by HFNPYRH to levels similar as the $\alpha_2\beta_3\gamma_2$ receptor (**Figure 21A**, dotted bar). Both $\alpha_2\beta_3$ receptors with tricine, and $\alpha_2\beta_3\gamma_2$ receptors, displayed a greater inhibitory effect in response to HFNPYRH than the $\alpha_1\beta_2\gamma_2$ receptor (**Figure 21A**, hollow bar; see figure legend for statistics). HFNPYRH produced no effect on channel function when applied alone, as seen by no change in baseline current during a 30 second pre-application (**Figure 21B**) prior to co-application with GABA.

100μM HFNPYRH produced slight enhancement of the α_1 glycine receptor (**Figure 22A**, solid black bar and **Figure 22B**, first three currents), but this enhancement was no longer visible in the presence of 2.5mM tricine (**Figure 22A**, hollow bar and **Figure 22B**, last three currents). Additionally, application of peptide HFNPYRH to the zinc-insensitive receptor mutant α_1 W170S also produced no enhancement of receptor currents; instead a slight reduction in receptor function was observed (**Figure 22A**, solid grey bar).

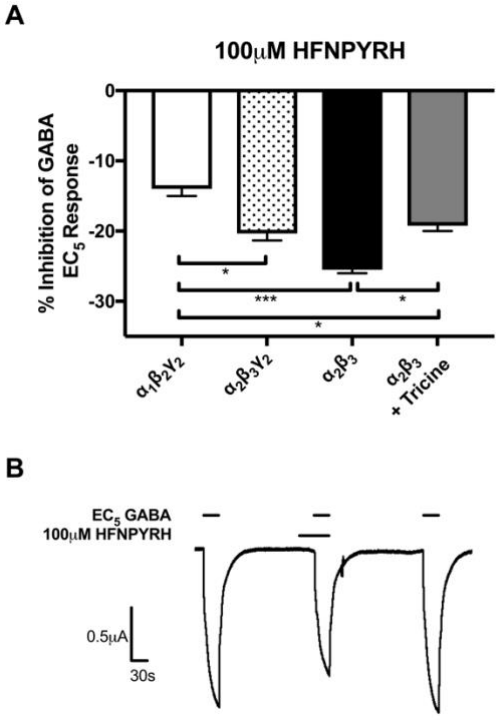

Figure 21. Peptide HFNPYRH decreases $GABA_A$ receptor function.

A. There was a significant effect of mutation/treatment in their responses to HFNPYRH; one way ANOVA [$F(3,10)=11.67$, $p<0.01$], with a Tukey's post hoc test showing specific differences. 100μM HFNPYRH decreased $\alpha_1\beta_2\gamma_2$ (hollow bar) $\alpha_2\beta_3\gamma_2$ (dotted bar) and $\alpha_2\beta_3$ (solid black bar) GABA function, with $\alpha_2\beta_3\gamma_2$ and $\alpha_2\beta_3$ receptors showing a greater decrease than $\alpha_1\beta_2\gamma_2$ receptors. The addition of tricine significantly decreased the amount of inhibition exhibited by $\alpha_2\beta_3$ $GABA_A$ receptors (solid gray bar), but the inhibition was still greater than that observed in $\alpha_1\beta_2\gamma_2$ receptors. (*, $p<0.05$; ***, $p<0.001$). Each bar represents the average from at least three oocytes from at least two different frogs, - the standard error of the mean. **B**. Representative tracing of an $\alpha_2\beta_3\gamma_2$ expressing oocyte's response to 100μM HFNPYRH. The peptide was pre-applied for 30 seconds prior to co-application of the peptide with EC_5 GABA.

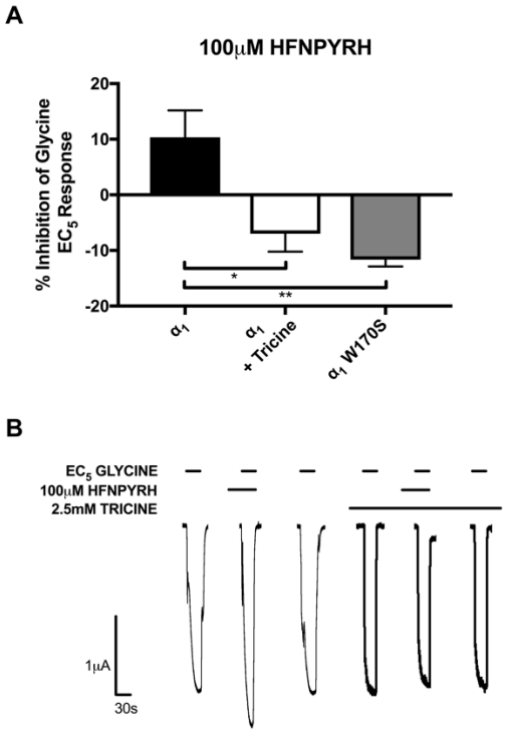

Figure 22. Presence of contaminants in peptide HFNPYRH may mask weak inhibitory effects at the α_1 glycine receptor.

A. While 100μM HFNPYRH appeared to have weak positive modulatory effects at the α_1 glycine receptor (solid black bar), this effect was reversed in the presence of 2.5mM tricine (hollow bar) and in the zinc-insensitive glycine receptor mutant, α_1 W170S (solid gray bar). One-way ANOVA revealed a significant effect of treatment/mutation [$F(2,6)=11.44$, $p<0.01$], with a Tukey's post-hoc test showing significant differences in the response of α_1 receptors, compared to α_1 receptors in the presence of tricine and α_1 W170S receptors (*,$p<0.05$; **, $p<0.01$). Bars represent the average of three oocytes from at least two frogs, with error bars representing the standard error of the mean. **B**. Representative tracing of 100μM HFNPYRH effects on α_1 glycine receptors in the absence (left three currents) and presence of 2.5mM tricine (right three currents). HFNPYRH was pre-applied for 30 seconds prior to a 30 second co-application with glycine. The positive modulatory effect or HFNPYRH disappears in the presence of tricine.

4.3.3 ACTIONS OF PEPTIDE WVPQRHQ ON GABA$_A$ AND GLYCINE RECEPTORS

100μM WVPQRHQ weakly inhibited $\alpha_1\beta_2\gamma_2$ (**Figure 23A**, hollow bar) and $\alpha_2\beta_3\gamma_2$ (**Figure 23A**, dotted bar) GABA$_A$ receptors to a similar extent. $\alpha_2\beta_3$ receptors (**Figure 23A**, solid black bar) showed increased inhibition by WVPQRHQ compared to $\alpha_1\beta_2\gamma_2$ and $\alpha_2\beta_3\gamma_2$ receptors, however the addition of 2.5mM tricine to $\alpha_2\beta_3$ receptors reverted inhibitory levels back to that of $\alpha_1\beta_2\gamma_2$ and $\alpha_2\beta_3\gamma_2$ receptors (see **Figure 23** legend for statistics). There was no change in baseline current when WVPQRHQ was applied alone, and the inhibitory effects of the peptide were only seen when the peptide was applied in addition to GABA EC_5 (**Figure 23B**).

100μM WVPQRHQ enhanced α_1 glycine receptor function by 33.3 ± 1.8 % (**Figure 24A**, solid black bar and **Figure 24B**, first three tracings) but no modulation of receptor function was observed in the presence of tricine (**Figure 24A**, only error bar visible and **Figure 24B**, last three tracings).

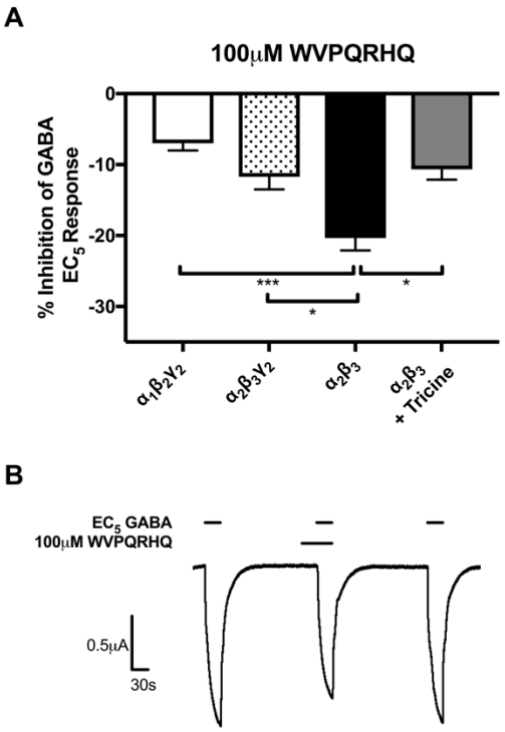

Figure 23. Peptide WVPQRH has a weak inhibitory effect on $GABA_A$ receptors.

A. WVPQRH produced significantly different effects in different $GABA_A$ receptor conditions: one way ANOVA [$F(3,11)=5.691$, $p<0.02$], followed by Tukey's post hoc test. 100μM WVPQRH similarly inhibited $\alpha_1\beta_2\gamma_2$ (hollow bar), $\alpha_2\beta_3\gamma_2$ (dotted bar) and $\alpha_2\beta_3$ receptors in the presence of tricine (solid gray bar) but all of these receptor subtypes differed significantly from $\alpha_2\beta_3$ receptors in the absence of tricine (solid black bar) (*, $p<0.05$). Bars represent the mean – the standard error of the mean from at least three oocytes from two frogs. **B**. representative tracing showing the weak inhibitory effect of 100μM WVPQRHQ on $\alpha_2\beta_3\gamma_2$ $GABA_A$ receptors when co-applied with GABA EC_5.

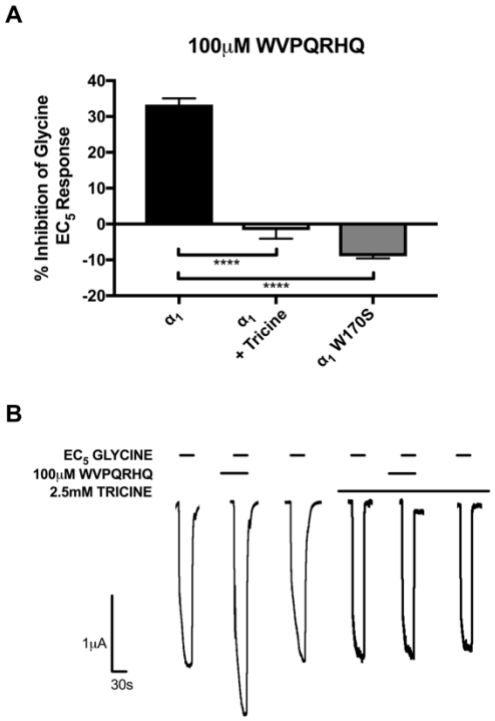

Figure 24. Positive modulatory effects of WVPQRHQ on α_1 glycine receptors disappear in the presence of tricine.

A. The effect of 100uM WVPQRHQ on α_1 glycine receptors in the absence of 2.5 mM tricine (solid black bar) is significantly enhanced compared to α_1 receptors in the presence of tricine (middle column, only error bar visible) and α_1 W170S mutant receptors (solid grey bar), one way ANOVA [$F(2,6)=166.5$, $p<0.0001$] followed by Tukey's post hoc test (****, $p<0.0001$). Each bar represents the data from three oocytes ± the standard error of the mean. **B.** Representative tracing of 100μM WVPQRHQ effects on α_1 glycine receptors in the absence (left three currents) and presence (right three currents) of 2.5 mM tricine. The presence of tricine eliminated the positive modulatory effects of WVPQRHQ seen on α_1 glycine receptors when WVPQRHQ was co-applied with glycine EC_5.

4.3.4 ACTIONS OF PEPTIDE TVQHLHR ON GLYCINE AND GABAA RECEPTORS

100μM TVQHLHR weakly inhibited $\alpha_1\beta_2\gamma_2$ and $\alpha_2\beta_3\gamma_2$ receptors to a similar extent (**Figure 25A** hollow bar and dotted bar, respectively). $\alpha_2\beta_3$ receptors (**Figure 25A**, filled black bar) appeared to be inhibited to a greater extent than the $\alpha_1\beta_2\gamma_2$ receptors, but not $\alpha_2\beta_3\gamma_2$ receptor. The presence of 2.5mM tricine reduced the extent of inhibition produced by $\alpha_2\beta_3$ receptors (**Figure 25A**, filled grey bars) back to levels not significantly different than that produced by $\alpha_1\beta_2\gamma_2$ receptors (see **Figure 25** legend for statistics). As with the other peptides, TVQHLHR had to be co-applied with EC_5 GABA to see effects, with no effect seen with the application of TVQHLHR alone, as seen by no change in baseline current during the pre-application of TVQHLHR (**Figure 25B**).

Only a slight positive modulatory effect was observed when 100μM TVQHLHR was co-applied with glycine EC_5 to the α_1 receptor (**Figure 26A**, filled black bar and **Figure 26B**, first three tracings). This positive modulation was significantly decreased in the presence of 2.5mM tricine (**Figure 26A**, hollow bar and **Figure 26B**, last three tracings) and in the α_1 W170S mutant receptor (**Figure 26A**, solid grey bar).

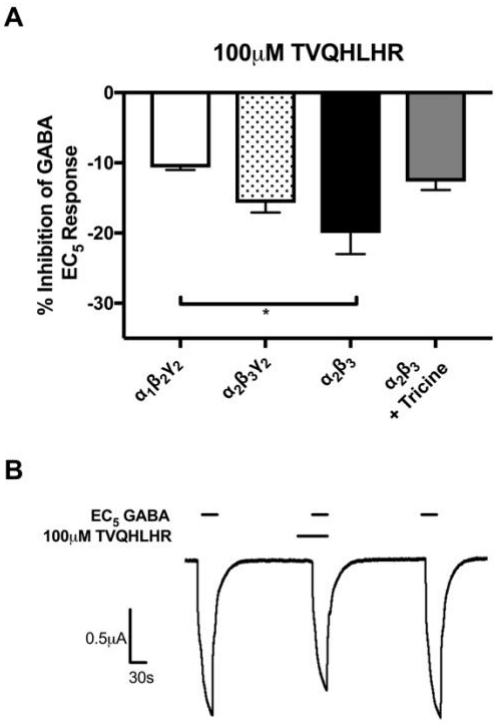

Figure 25. Peptide TVQHLHR negatively modulates $GABA_A$ receptors.

A. The four GABA receptor conditions, $\alpha_1\beta_2\gamma_2$ (hollow bar), $\alpha_2\beta_3\gamma_2$ (dotted bar), $\alpha_2\beta_3$ (solid black bar) and $\alpha_2\beta_3$ with tricine (solid gray bar) differed significantly in their receptor responses to 100μM TVQHLHR, one way ANOVA [$F(3,11)=4.36$, $p<0.05$] with Tukey's post hoc test (*, $p<0.05$). Each bar represents data from at least three oocytes – the standard error of the mean.
B. representative tracing of 100μM TVQHLHR actions on $\alpha_2\beta_3\gamma_2$ $GABA_A$ receptors. The co-application of TVQHLHR with GABA EC_5 produces a slight reduction in receptor function, but no current is produced by TVQHLHR in the absence of GABA EC_5, as seen by the absence of a response during a 30 second pre-application of TVQHLHR without GABA EC_5.

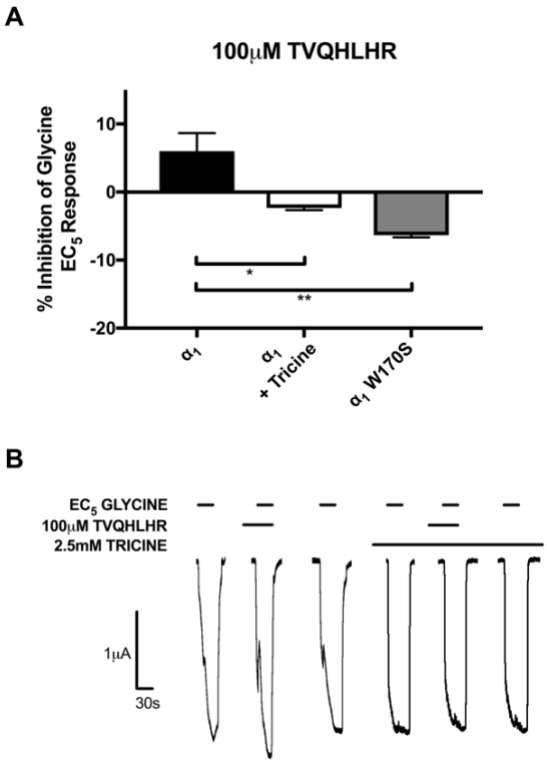

Figure 26. Weak positive modulatory effect of 100μM TVQHLHR on α_1 glycine receptors is absent in the presence of tricine and in the α_1 W170S receptor mutant.

A. α_1 glycine receptors (solid black bar), α_1 glycine receptors in the presence of 2.5mM tricine (hollow bar) and mutant α_1 W170S glycine receptors (solid grey bar) differed significantly in their responses to 100μM TVQHLHR, one way ANOVA [$F(2,6)=16.45$, $p<0.01$] with Tukey's post hoc (*, $p<0.05$; **, $p<0.01$).Each bar represents data from three oocytes, + or – the standard error of the mean. In the case of α_1 receptors in the presence of tricine the error is too small to depict. **B.** Representative tracing of the effects of 100μM TVQHLHR on α_1 glycine receptors in the absence (left three currents) and presence (right three currents) of 2.5mM tricine. 100μM TVQHLHR produces a small potentiation in the α_1 glycine receptor when co-applied with glycine EC_5, but this is eliminated in the presence of tricine. The 30 second pre-application of TVQHLHR prior to the co-application with glycine produced no change in baseline current.

4.4 Discussion

In this chapter we demonstrated the use of phage display technology to identify peptides capable of changing $GABA_A$ receptor function. While previous studies in our lab did not use a control screen (panning twice against un-transfected cells), they did employ a negative selection process against cells expressing a receptor they did not want to select for before the positive selection step against cells expressing the desired target (Tipps et al., 2010; Cornelison et al., 2016). However, this study found that little selectivity resulted despite including this negative selection step, probably due to the similarity between the receptor in the negative selection step and receptor in the positive selection step. The GABA α_1 and α_2 subunits are 81% similar, and β_2 and β_3 are 74% similar. Due to the similarity found between the $\alpha_1\beta_2\gamma_2$ and $\alpha_2\beta_3\gamma_2$ receptors, it could be possible that by stringently selecting against $\alpha_1\beta_2\gamma_2$ receptors we would eliminate peptides that bind both receptor types, but have a greater effect on $\alpha_2\beta_3\gamma_2$ over $\alpha_1\beta_2\gamma_2$ receptors. Additionally, peptide libraries have sequence biases, resulting in certain peptides appearing round after round despite no selection for the target occurring (Zade et al., 2017). Hence, we felt it was important to include the control experiment, where peptides were panned twice against un-transfected cells.

In our panning, we found 23 peptides that appeared in both the control and the $\alpha_2\beta_3\gamma_2$ selection experiment, while we found only 11 peptides that appeared solely in the $\alpha_2\beta_3\gamma_2$ selection experiment (**Table 1**). Previous phage display experiments in the lab found that the most efficacious peptides had specific consensus sequences, and after analyzing traits of our identified peptides we noticed the presence of positive amino acids in the tail end of some peptides (Tipps et al., 2010; Cornelison et al., 2017). This trait was observed only in peptides found in the $\alpha_2\beta_3\gamma_2$ selective experiment, not in the control experiment. All three peptides tested with two-electrode voltage clamp electrophysiology inhibited $\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_3\gamma_2$ and $\alpha_2\beta_3$ receptors, but to differing

extents, with HFNPYRH having the greatest effect. Due to the selection of these peptides based on a consensus sequence, it is not surprising that they all acted as negative modulators.

Peptide HFNPYRH displayed selectivity for $\alpha_2\beta_3\gamma_2$ $GABA_A$ receptors over $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors, as it inhibited $\alpha_2\beta_3\gamma_2$ receptors to a greater extent (**Figure 21A** dotted bar versus hollow bar). To probe if the γ_2 subunit was necessary for the actions of HFNPYRH, we tested the actions of the peptide on $\alpha_2\beta_3$ receptors. The $\alpha_2\beta_3$ $GABA_A$ receptors were still inhibited by HFNPYRH, indicating that the γ_2 subunit is not necessary for this peptide's actions (**Figure 21A**, solid black bar). The $\alpha_2\beta_3$ $GABA_A$ receptors are sensitive to low levels of zinc, with zinc acting as an allosteric inhibitor of receptor function, while $GABA_A$ receptors containing the γ_2 subunit are only sensitive to high levels of zinc. Our lab has previously identified that zinc is a common contaminant in labware, buffers, chemicals, and synthetic peptides (Cornelison and Mihic, 2014; Cornelison et al., 2016). We therefore wanted to see if the effect of the peptide seen on these receptors was possibly due to zinc. Chelating out zinc with tricine reduced the amount that HFNYPYRH inhibited $\alpha_2\beta_3$ receptors (**Figure 21A**, filled grey bar) to levels not significantly different than that of $\alpha_2\beta_3\gamma_2$ receptors. To further test if the peptide had zinc contamination present, we tested peptide HFNYPYRH on glycine receptors. Glycine receptors are also sensitive to low levels of zinc, but in contrast to $\alpha\beta$ $GABA_A$ receptors, they are potentiated by low levels of zinc. 100 μM HFNPYRH produced a slight potentiation in the α_1 receptor, but this was reversed upon the addition of tricine (**Figure 22A** and **Figure 22B**). This indicated the possibility that there was zinc contamination in the peptide. Although the addition of tricine resulted in the peptide appearing slightly inhibitory when combined with glycine EC_5, it is difficult to tell if this is just noise or if the peptide is truly having a weak effect. While specific modulators for GABA or glycine receptors exist, it should be noted that other modulators, such as ethanol and zinc, are able to act on both receptors. This is not surprising given the high amino acid sequence homology between the two receptors. Further

evidence of contaminating levels of zinc is peptide HFNPYRH's actions on the mutant α_1 W170S receptor. The W170S receptor, in which the glycine receptor α_1 subunit tryptophan residue 170 has been mutated to a serine, is insensitive to the potentiating concentrations of zinc (Cornelison et al., 2017). The similar actions of HFNPYRH on the W170S receptors and the α_1 glycine receptor in the presence of tricine (**Figure 22A**) further suggests that there are contaminating levels of zinc in the peptide.

The second peptide, WVPQRHQ, exhibited no selectivity for $\alpha_2\beta_3\gamma_2$ receptors over $\alpha_1\beta_2\gamma_2$ receptors (**Figure 23A**, hollow and dotted bar). However, the $\alpha_2\beta_3$ receptor, lacking a γ_2 subunit, produced significantly more inhibition (**Figure 23A**, solid black bar). This would suggest that the γ_2 was not necessary, or perhaps even hindered the peptide from working effectively. However, in the presence of tricine, the level of inhibition by WVPQRHQ was reduced to levels that do not differ from $\alpha_1\beta_2\gamma_2$ or $\alpha_2\beta_3\gamma_2$ receptors, pointing at the possibility of zinc contamination in this peptide (**Figure 23A**, filled gray bar). The possibility of contaminating zinc was further supported by the positive modulation of the α_1 glycine receptor, which disappeared in the presence of tricine and on the W170S receptor (**Figure 24A** and **Figure 24B**).

The third peptide, TVQHLHR, also exhibited no selectivity for the $\alpha_2\beta_3\gamma_2$ receptor over the $\alpha_1\beta_2\gamma_2$ (**Figure 25A**, hollow bar and dotted bar). Both $\alpha_1\beta_2\gamma_2$ and $\alpha_2\beta_3\gamma_2$ receptors were inhibited by TVQHLHR, and although the data appears as though there may be a greater enhancement at $\alpha_2\beta_3\gamma_2$ receptors than at $\alpha_1\beta_2\gamma_2$ receptors, this trend did not result in a significant difference. Inhibition of $\alpha_2\beta_3$ receptors by TVQHLHR was significantly greater compared to inhibition of $\alpha_1\beta_2\gamma_2$ receptors, but this was not the case in the presence of tricine (**Figure 25A**, solid black and grey bar). This suggested that TVQHLHR may also have traces of contaminating zinc, resulting in a greater apparent inhibition in the absence of tricine. TVQHLHR actions on the

α_1 glycine receptor and glycine receptor mutants suggested that perhaps a small amount of zinc was present (**Figure 25B**).

The varied effects of the three peptides at the glycine receptor indicates different levels of contaminating zinc (**Figure 22A**, **Figure 24A** and **Figure 26A**). However, the level of predicted zinc does not correlate with how effectively the peptide inhibits the $GABA_A$ receptor. For example, peptide WVPQRHQ (**Figure 23A**, filled black bar) appears to have the most contaminating zinc present, as indicated by it producing the most enhancement at α_1 glycine receptors; however, it has a slightly weaker inhibitory response at $\alpha_2\beta_3$ receptors than peptide HFNPYRH (**Figure 21A**, filled black bar). This indicates that the peptides are producing an inhibitory effect, rather than contaminating zinc producing an effect.

There was no effect of the peptides when applied alone in the absence of an agonist, indicating that the peptides are not acting as direct agonists of the $GABA_A$ and glycine receptor. However, it is unclear if the three peptides investigated in this study are in fact negative modulators, or competitive antagonists. In the case of known inverse agonists, for example Ro 15-4513, the competition of Ro 15-4513 with a benzodiazepine site antagonist (flumazenil) reveals its site of action, the benzodiazepine site. However, these three peptides do not require the γ_2 subunit in order to exert their actions, suggesting that they do not act at the benzodiazepine site, as benzodiazepines do require the γ_2 subunit. Therefore, the actions of the peptide would not be competed by flumazenil, and at this time the distinction between inverse agonist and competitive antagonist cannot be made.

It is important to address the clinical or experimental usefulness of the three peptides studied. While one may think that an inverse agonist acting weakly at $GABA_A$ receptors would be

of no use, and induce anxiety and insomnia rather than alleviate it, the inverse agonist Ro 15-4513 has proven to be a powerful tool, both experimentally and as a potential therapeutic. Ro 15-4513 was originally developed as an antidote to alcohol intoxication, as it can block the effects of alcohol as well as the effects of benzodiazepines (Bonetti et al., 1988; June and Lewis, 1994). While pharmacokinetic and safety concerns prevented it from getting Food and Drug Administration approval, it provided a template for which future inverse agonists may have therapeutic use. Ro 15-4513 has also been useful in other applications, such as in positron emission tomography (Inoue et al., 1992; Maeda et al., 2003; Lingford-Hughes et al., 2012; Lingford-Hughes et al., 2016). Thus, although the peptides we have identified were opposite in action that what our study aimed to achieve, they still have potential to act themselves, or as a template, for future experimental or clinical use.

Another concern of this study may be the high concentrations of peptide used to probe GABA receptor function. 100μM exceeds the concentration that would be of therapeutic use, and while lower concentrations were tested (down to 10μM), the peptides identified are of low potency. However, this is not unusual when compared to other phage display pannings that were completed in our lab (Tipps et al., 2010; Cornelison et al., 2016). This low potency might be due to an avidity effect, where polyvalent (five identical peptides per phage) display on the bacteriophage may be causing a higher apparent affinity compared to what the actual affinity of one peptide to one receptor (Lowman, 1997). Additionally, the potency in oocytes might not necessarily be equal to what would therapeutically benefit a human. For example, the human blood alcohol concentration achieved at the legal driving limit (80 mg/dL) is equal to 17.4mM. However, in most $GABA_A$ receptors, little effect of ethanol is seen on oocytes until ethanol concentrations exceed 50mM (Mihic et al., 1997; Ueno et al., 1999). Yet the culmination of small effects on various receptors is thought to contribute to alcohol induced impairment (Wallner et al., 2006). According to this

rationale, the peptides that we tested might produce a therapeutic effect at lower concentrations in vivo.

This study aimed to find peptides via phage display capable of selectively potentiating the $\alpha_2\beta_3\gamma_2$ $GABA_A$ receptor. A peptide capable acting as a positive modulator at this receptor may have therapeutic benefit for the treatment of anxiety. Our study identified three peptides that inhibited receptor function, either as a competitive antagonist at the GABA binding site or as an inverse agonist at a novel allosteric site While these compounds would produce rather than alleviate anxiety, these peptides may be useful in elucidating molecular mechanisms of other $GABA_A$ receptor acting compounds, or antagonize the effects of other agonists or modulators. Moreover, this study acts as a proof-of-principal that phage display technology can be employed to find $\alpha_2\beta_3\gamma_2$ selective compounds.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Central nervous system disorders involve an imbalance between excitatory and inhibitory signaling in the brain, which is in part mediated through ligand-gated ion channels. However, only 7% of FDA-approved drugs target ligand-gated ion channels, and over half of these drugs were approved before 1990 (Santos et al., 2017). This statistic is surprising, given the range of disorders in which ligand-gated ion channels have been implicated. This paucity in the recent development of drugs targeting ligand-gated ion channels may be a result of the inability to find subunit-selective compounds, or the move towards more downstream targets in the hope of bypassing off-target effects. While subunit selectivity may be hard to achieve when comparing agonist binding sites between two subtypes of receptors, there may be numerous allosteric binding sites on a receptor that do differ between subtypes. Since ligand-gated ion channels are transmembrane proteins made up of four (glutamate ion channels) or five (cys-loop channels) subunits, this provides multiple binding sites within and between adjacent subunits. Additionally, besides providing selectivity, allosteric modulators may also have the property to act as a dimmer switch; rather than an agonist (on switch) or antagonist (off switch); an allosteric modulator can tweak normal function of the receptor rather than produce a drastic and possible detrimental effect (Abdel-Magdid, 2015). This ability to tweak receptor function rather than change it completely may be key when targeting ligand-gated ion channels, since they are implicated in so many cellular functions.

5.1 Endogenous Compounds as Modulators of Ligand-Gated Ion Channel Function

Chapter two detailed the ability of ketone bodies to modulate NMDA, $GABA_A$ and glycine receptor function. My findings demonstrate that endogenous compounds can act as allosteric modulators. Other endogenous substances, such as zinc, neurosteroids and polyamines, are also capable of modulating ligand-gated ion channel function. These modulators are believed to act at different sites, can produce opposing effects at the same receptor (e.g. glycine receptor's biphasic response to zinc), and have opposing functional effects at different receptors (e.g. β-hydroxybutyric acid decreases NMDA receptor function, leading to decreased excitation, but also decreases GABA receptor function, leading to decreased inhibition). This demonstrates the diversity of possible binding sites for allosteric modulators, but also demonstrates that the actions of an allosteric modulator are going to depend on brain region, receptor type, and differences in affinity of the modulator for a receptor. In the case of β-hydroxybutyric acid, NMDA receptors have a higher affinity for the compound than $GABA_A$ receptors, suggesting that in a brain region expressing both receptors at equal amounts, the net effect of β-hydroxybutyric acid would be a decrease in excitation. Given the literature surrounding the therapeutic benefits of ketogenesis, from preventing epileptic seizures to alleviating alcohol withdrawal symptoms, the actions of β-hydroxybutyric acid on NMDA receptors may be key in producing these effects. Defining the allosteric binding sites of ketone body action at NMDA, $GABA_A$ and glycine receptor function may be key in producing novel compounds that might have the ability to produce the same therapeutic benefit as ketogenesis.

The importance of replicating data and ensuring that the necessary control experiments were performed is highlighted in chapter two. While we were able to replicate work from several

other studies, we were not able to replicate the work of Yang et al. (2007) which detailed positive allosteric effects of ketone bodies at GABA and glycine receptors, and no effect of β-hydroxybutyric acid on NMDA receptors. These contradictory results may be a consequence of not using adequate controls. Specifically, whenever investigating new compounds that may exert actions glycine and NMDA receptors, it is important to control for contaminating zinc in the compounds themselves as well as in labware and buffers.

While the benefits of β-hydroxybutyric acid in reducing seizures in rodent models has been described, it would be interesting to see similar studies conducted for other therapeutic indications. Fasting or specific low carbohydrate diets for short periods of time in rodents may not produce ketone body levels capable of producing a therapeutic effect, so direct injection of β-hydroybutyric acid may provide more insight into the clinical potential of ketone bodies/ketone body mimetics. Comparing the therapeutic benefit of β-hydroxybutyric acid alone and in combination with NMDA or GABA receptor antagonists may help decipher if the NMDA and/or GABA receptors are mediator(s) of the therapeutic effect.

5.2 Understanding the Molecular Mechanism of Benzodiazepine Action

Despite benzodiazepines being well characterized based on their binding site and their separate actions on different $GABA_A$ receptor subtypes, a lot remains unknown about how receptor binding results in an enhancement of receptor function. Investigating the structural mechanisms that underlie allosteric modulation is key for future drug development, and for understanding interactions between multiple drugs acting at the same receptor.

Other studies have demonstrated that benzodiazepine-site positive modulators induce different structural rearrangements in the $GABA_A$ receptor compared to negative modulators that act on the same site (Boileau and Czajkowski, 1999; Hanson and Czajkowski, 2008). In agreement with this, we found that the actions of the benzodiazepine-site inverse agonist Ro 15-453 are not impacted by the aspartic acid 75 - lysine 104 electrostatic interaction that we hypothesize is part of the structural rearrangement produced by benzodiazepine site positive modulators. While other studies suggest that the α_1 $GABA_A$ receptor selective modulator zolpidem produces a different conformational change in the receptor compared to non-selective benzodiazepines, our data suggest that they might have some overlap in the conformational rearrangements that they produce.

Interestingly, ethanol and allopregnanolone, believed to act at different sites than the benzodiazepine site, were not affected by the crosslinking of α_1 lysine 104 and γ_2 aspartic acid 75. Our data suggests that ethanol and allopregnanolone produce different conformational changes in the receptor than benzodiazepines, and act in an additive manner to benzodiazepines. This is of clinical importance because ethanol and benzodiazepines are often co-abused and produce enhanced impairment when taken in combination (Vanover et al., 1998; Chan, 1984).

The electrostatic interaction described in chapter three is likely to be only a part of a wave of conformational rearrangements that occur in the receptor after binding either agonists or allosteric modulators acting at the benzodiazepine site. Many other electrostatic interactions, and non-electrostatic interactions, probably contribute to the enhancement of receptor function, and further investigation of these rearrangements will enhance understanding of allosteric modulation and the design of future therapeutics. Additionally, our study demonstrated that structural models of the $GABA_A$ receptor, while not perfect, can provide a useful starting point in untangling the conformational wave produced by allosteric modulators. Improving these structural models

through further mutagenesis studies will provide a stronger basis for rational drug design at ligand-gated ion channels.

5.3 Identification of New Allosteric Modulators via Phage Display Technology

In chapter four we utilized genetically modified bacteriophage to pan peptides against a desired target, the $\alpha_2\beta_3\gamma_2$ receptor. We chose this target due to its therapeutic potential in alleviating anxiety without producing sedative effects. Previous work in our lab (Tipps et al., 2010; Cornelison et al., 2017) demonstrated that peptides capable of modifying the glycine receptor (a target for alcoholism, pain and epilepsy) could be identified using phage display, albeit with low affinity and low selectivity between glycine receptor subtypes. However, since the $\alpha_2\beta_3\gamma_2$ and $\alpha_1\beta_2\gamma_2$ receptor have less homology between them than two subtypes of the glycine receptor, we felt that it may be possible to achieve selectivity for the $\alpha_2\beta_3\gamma_2$ receptor.

Screening of identified peptides with a shared consensus sequence identified three peptides that were capable of negatively modifying the receptor. The first peptide, HFNPYRH, was the most efficacious and had selectivity for $\alpha_2\beta_3\gamma_2$ receptors over $\alpha_1\beta_2\gamma_2$ receptors. The other two peptides were not as efficacious and had poor selectivity. Applying a chelator and screening the peptides against wildtype and mutant glycine receptors ensured that our effects were real and not due to contaminating zinc. However, since the peptides we identified were negative modulators of the $GABA_A$ receptor, they would likely act in an anxiogenic rather than an anxiolytic manner in vivo. Additional phage display pannings and electrophysiological screening of other peptides that appeared in our initial panning may result in the identification of a selective $\alpha_2\beta_3\gamma_2$ positive modulator capable of producing anxiolytic effects without sedation. Additionally, including a

negative selection step against unwanted targets (e.g. the $\alpha_1\beta_2\gamma_2$ receptor) may provide enhanced selectivity than what was seen in our study.

Chapter four of this dissertation provides a proof-of-concept for the use of phage display to identify allosteric modulators of ligand gated ion channels, for a variety of indications mentioned in chapter one. The low potency of peptides identified in this study and previous studies should not dissuade one from the use of phage display to identify compounds. Rather, low potency peptides that are identified through phage display may provide a starting point to develop a higher affinity compound. This could involve the design of new phage display libraries, where variations of the initial identified peptide are panned against receptors.

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